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Evaluation of microbial indicators to distinguish sources of pollution in groundwater.

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EVALUATION OF MICROBIAL INDICATORS TO DISTINGUISH SOURCES OF POLLUTION IN GROUNDWATER

by

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Abstract

In this study three different Microbial Source Tracking methods (MST) have been evaluated in laboratory experiments with the objective of determining which is more effective and reliable in distinguishing between human and animal sources of fecal contamination in groundwater.

A preliminary literature review of all the MST methods applicable for surface and ground water has been done, and the use of microbiological indicators has been selected. The methods selected involved the enumeration of Fecal Coliforms (FC), Fecal Streptococci (FS), *Clostridium perfringens* (CP) and Human Bifidobacteria (SFB), parameters which are generally defined as Bacterial Indicators (BI). The first method consisted of calculating the FC/FS ratio, the values of which can be related to animal or human sources, while the second method consisted of the enumeration of CP, which in some studies was associated with animal sources of fecal pollution. Finally, the third method enumerated levels of SFB, which are associated exclusively with human sources of fecal pollution.

In the first phase of this research the three MST methods have been tested on samples of manure and of wastewaters of known origin in order to assess the effectiveness in discriminating the source of fecal contamination.

In the second phase, in order to assess the effects of time on the BI, three experiments simulating a typical Southern-Ontarian groundwater environment (here defined as GSME experiments), was set up. The concentration of the BI was determined over a period of 60 days; the ability

of the MST methods to predict the source of contamination was tested over the same period.

In the third phase, three GSME experiments were organized with the objective of determining the die-off coefficients for the pure species of *C. perfringens* and *B. adolescentis* (the most important among the SFB), and for human Bifidobacteria (SFB) isolated from septic system waste. The determination of these parameters, which are not retrievable in the literature, is fundamentally important to evaluate how these BI can perform as source specific fecal indicators.

The last phase of this study consisted of applying the MST methods to samples of groundwater taken from wells located in rural areas. The objective of this phase was to use the MST methods with real samples, and possibly to identify differences in performance with respect to the previous phases.

The results obtained indicate that, among the methods selected, only the determination of SFB can be considered reliable. Nevertheless, it was observed that this BI was detectable only for a short period after the contamination event, and therefore it appears that it is applicable only in cases of recent pollution. This factor is a considerable limit to the use of this MST method.

On the other hand, this study has reconfirmed that CP can be very useful as a general indicator of the timing of a fecal contamination event, rather than as a source specific indicator.

Dedication

Come segno di infinita gratitudine, dedico questo lavoro a tutte le meravigliose persone che qui in Canada si sono prodigate nell'offrirmi sostegno, amicizia e buona compagnia.

Dedico inoltre questa tesi a chi in Italia ha sentito la mia mancanza durante questo periodo di distacco, ed in particolare a Michela, che sempre mi sostiene.

Acknowledgements

I would like to thank my advisors Dr N. Biswas, Dr A. Hubberstey and Dr J.K. Bewtra for their constant support and guidance during this research. A special thank to Dr J. Laird, Mr. Eddy Manzocco, Mr. Peter Bziuk and Mr. D. Armstrong, for the samples provided. My gratitude goes also to Miss Tenley Noone, Mrs. Usha Jacob, Mrs. Monica Murphey and Ms Carol for their assistance.

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List of acronyms used

ARA	Antimicrobial Resistance Analysis
ATCC	American Type Culture Collection
Ba	*Bifidobacterium adolescentis*
Baγ, Baδ	groundwater simulated microenvironment inoculated with *B. adolescentis* (pure species) culture (I and II replicate)
BI	Bacterial Indicator
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
CP	*Clostridium perfringens*
Cpα, Cpβ	groundwater simulated microenvironment inoculated with *C. perfringens* (pure species) culture (I and II replicate)
FC	Fecal coliforms
FC/FS	Fecal Coliforms, Fecal Streptococci ratio
FS	Fecal streptococci
FWAs	Fluorescent whitening agents
GSME	Groundwater Simulated Microenvironment
HBSA	Human Bifidobacteria Sorbitol-fermenting agar
LBAs	Long-chain alkylbenzenes
MAR	Multiple antibiotics resistance analysis
mCP	*Clostridium perfringens* medium
mCPm	*Clostridium perfringens* medium modified
MST	Microbial Source Tracking methods

P1	groundwater simulated microenvironment inoculated with fresh pig manure
P2	groundwater simulated microenvironment inoculated with manure stored in a cesspit of a pig farm
S	groundwater simulated microenvironment inoculated with wastewater from a septic system
SFB	Sorbitol Fermenting Bifidobacteria
SFB α, and β	groundwater simulated microenvironment inoculated with Sorbitol-fermenting Bifidobacteria culture, isolated for wastewaters of human origin (I and II replicate)
SFP	Shahidi-Ferguson perfringens Agar
STP	Sewage Treatment Plants
STPs	sodium tripolyphoshate
TC	Total coliforms
WTP	Wastewater Treatment Plant

Chapter 1

Introduction

The importance of water resources in modern society is emphasized by the renewed interest given by the international community to all the fundamental issues related to it. Besides the need of always increasing quantities of fresh water, the problem of maintaining a good quality is the main concern of water managers around the globe. Amongst all the available sources of drinking water, groundwater represents one of the main supplies, and this is particularly true in rural areas of North America, where groundwater represents the only source of drinking water for a large percentage of the population. Here, the quality of groundwater is threatened by a long list of pollutants, but probably waterborne pathogens are the most well know. In fact, during the first decade of the nineteenth century, when the breakthrough of microbiology was disclosing new insight on the cause of some feared diseases, it was established that some of the more notorious bacterial pathogens could be transmitted via contaminated water (CDC, 2000). Thereafter, a long list of other significant waterborne pathogens, including bacteria, viruses, helminths and protozoans, has been found to be transmissible via water.

Recent surveys are showing that, in a significant fraction of rural households, drinking water is not acceptable from the bacteriological stand point. This is in spite of all scientific evidences on the mechanism of infection

for waterborne pathogens and all the treatment technologies developed in order to maintain a good quality standard. This scenario does not apply only to the so called "developing countries", but it is alarmingly real also in North America, as reported in recent studies (Craun, 1992).

The increased pressure on water resources from all sectors of human society (residential, industrial and agricultural) puts enormous stress on public administrators and water managers, and unfortunately several waterborne outbreaks have occurred in the last decade in North America (CDC, 1994). In Canada, in particular, two remarkable outbreaks can be mentioned: the first happened in Walkerton - Ontario in May 2000, while the other in North Battleford - Saskatchewan in April 2001. Waterborne pathogens and microbial pollution in general feed the public concern for health and environmental risks. For this reason, the monitoring of microbial quality of drinking water is a pivotal activity in water resource management.

Pathogenic microorganisms found in surface or ground waters might be excreted by humans and animals who are infected with diseases, or who are simply carriers of a particular infection. The presence of these microbial pathogens can be directly detected; nevertheless it is convenient to use Bacterial Indicators (BI) because pathogens are usually low in number and are difficult to isolate. In this context, a BI is a surrogate of the presence of pathogen microbes; it gives an indirect "indication" on the potential presence of the pathogens although these are not directly detected. In order to be a good indicator, a microorganism should satisfy the following characteristics

(Maier, 2000):

a. it should be a member of the intestinal micro-flora of warm-blooded animals.
b. it should not be able to reproduce outside the host organism (or better, it should not be able to reproduce in the environment);
c. it must survive longer than the real pathogens in the environment;
d. it must be always present whenever there is fecal contamination;
e. its concentration should be greater than that of the pathogen; and
f. its isolation and quantification should be easier, faster and convenient when compared to that of the pathogens.

These criteria for the "Ideal Indicator Organism" are further discussed in Chapter 2; however, it is worthwhile to explain the logic underlying the convenience of using Bacterial Indicators than directly detecting the presence or absence of a pathogenic microorganism.

As has been previously mentioned, the maintenance of a good standard for water quality is the main concern of that part of the population that still relies on groundwater as a source for drinking purposes, and in particular there is an interest in maintaining a low level of risk of contracting an infection caused by waterborne pathogens. From the analysis of the mechanism of transmission of these infections the following pattern can be

evidenced:

a. excretion of the pathogen through the feces by an infected host (in this case a human being);
b. contamination of the water source by feces and potentially by the pathogen contained in the feces;
c. exposition to the pathogen through ingestion of the contaminated water by a second subject that can be infected by this pathogen; and finally
d. contraction of the disease by the second subject.

The cycle of transmission of the infection then restarts from the beginning (a new infected subject is in step a.). In order to keep the risk of infection to a minimum level, it is imperative to avoid the use of waters that have been contaminated by feces, even when there is no direct evidence of the presence of pathogens. In this case, the use of Bacterial Indicators is particularly convenient, because whenever the presence of these indicators in ground or surface water samples is determined; this is taken as a "signal" that pathogens associated with feces may also be present, while their absence should guarantee that water is free from disease-producing organisms of fecal origin. The organisms traditionally adopted as indicators of fecal pollution in waters are: Total Coliforms; Fecal Coliforms and Fecal Streptococci.

Recently, beside the traditional concern for fecal contamination and the related risks of waterborne outbreaks, there is also the need to identify and

distinguish the sources of fecal contamination (Sinton, 1998; Scott, 2002). The reason of this is the increasing stress of agricultural activities on water resources, and in particular of animal farming for meat production, that has reached an "industrial" dimension. In this case, the distinction of the source of contamination could be of great help to water managers. The other new concern that is distressing public health administrators is represented by the risk of contracting diseases transmittable by animals to humans, also called zoonoses. On the other hand, it should always be remembered that the identification of the sources of pollution is an indispensable information for the epidemiological study of waterborne pathogens. In this new context, there is the need to revisit and readapt the methods traditionally used for the determination of the microbiological quality in drinking water.

This thesis deals with microbiological methods that can be used to distinguish human from animal fecal contamination in groundwater.

1.1 – Objectives

This study arises from a practical problem recently faced in a rural area in Southwestern Ontario, where a well used for drinking water started showing fecal contamination. Because there are different potential sources of microbial pollution for that area; namely human sources, represented by septic systems, and animal sources, represented by the spreading of pig manure in the surrounding fields, it is difficult to ascertain which of these two can be the real cause of contamination using the information available. Hence, the objective of this study was the evaluation and comparison in

laboratory experiments of certain Microbial Source Tracking (MST) methods used to distinguish between human and animal fecal contaminations in groundwater. These methods can give the decisive indication of the source of pollution in case of ambiguity.

An attempt has also been made to try the selected MST methods with real samples of groundwater taken from two wells, although in this case there was no intention of identifying the source of contamination, but just to verify how these methods can perform in the field.

1.2 – Scope

The scope of this project was limited to a laboratory scale study. In order to assess the Microbial Source Tracking methods, the experimental activities were programmed in the following three phases:

I. Testing of the ability to distinguish between human and animal fecal contaminations with the selected MST methods in real samples.

II. Observation of the behavior of the Bacterial Indicators in a groundwater simulated micro-environment.

III. Determination, in a groundwater simulated micro-environment, of the persistence of *Clostridium perfringens* and *Bifidobacterium adolescentis* pure species, the two main BI investigated in this study.

IV. Testing of the methods on three water samples taken from two wells located in rural areas.

The experimental activities and the expected results are outlined in Figure 1.

Because of the assumption made, the results obtained should be considered valid only at a laboratory scale and under the specific conditions used in this study.

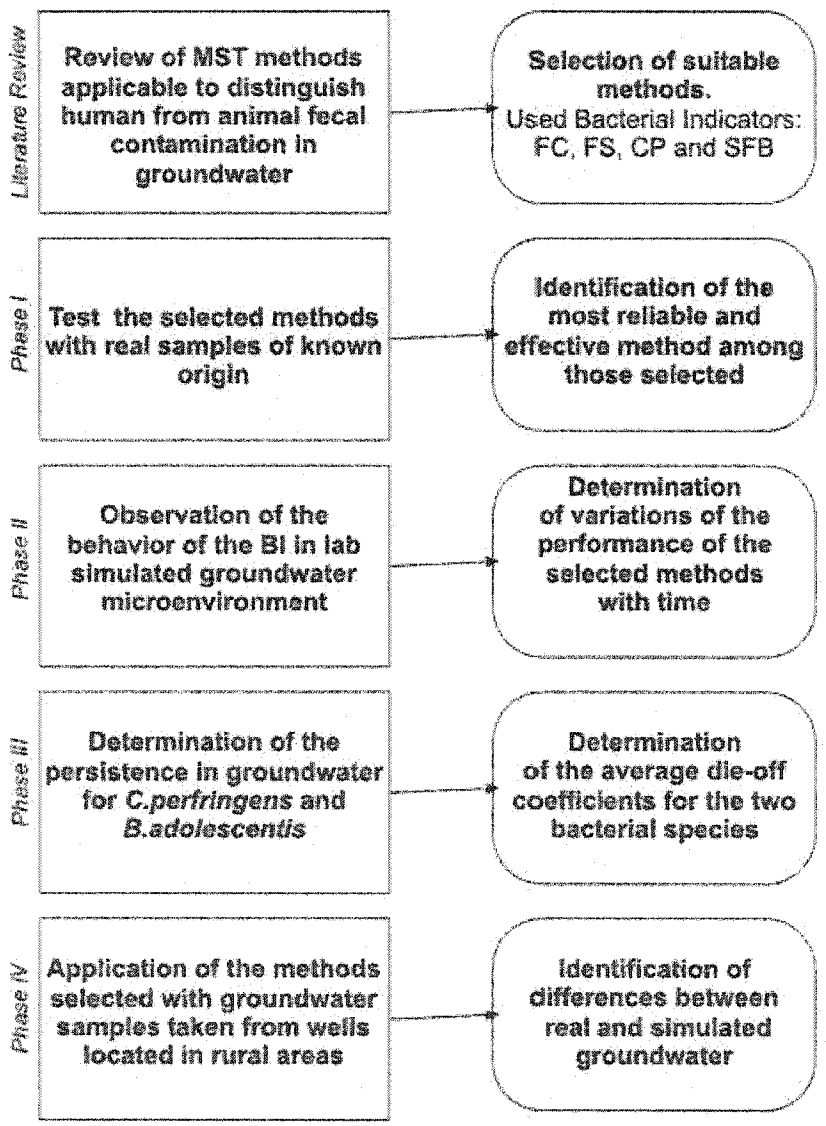


Figure 1.1 – Outline of the experimental activities

Chapter 2

Literature Review

2.1 – Groundwater Microbial Contamination

Traditionally, microbial water contamination has been associated to surface waters, in particular rivers, lakes and costal areas. In fact these water bodies can be directly affected by a source of fecal contamination since there is no "barrier" between the source and the final receiving body. Until recently, groundwater was considered to be protected from this type of contamination (CDC, 2000). There were three reasons for this common belief. First, it was assumed that the groundwater environment was not suitable for any kind of life; second, it was taken for granted that microbial contaminated water passing through the soil layers forming the aquifer was purified by filtration and by active subsurface processes; and finally, there was only a limited number of methods for sampling groundwater environments for bacteria and viruses. Therefore, there was very little concern for groundwater microbial pollution, and water managers operating in the field of groundwater resource were more concerned for other forms of contamination, such as heavy metals, petroleum derivates and synthetic organic compounds (Bitton, 1984). Nonetheless, in the last two decades new evidence has demonstrated the weakness inherent in these assumptions, feeding the interest for understanding subsurface microbiology, and in particular groundwater microbiology.

The occurrence of waterborne outbreaks associated with groundwater

microbial pollution in the last ten years has tested the conviction that groundwater is invulnerable to fecal pollution. In addition, the development of new microbiological detection methods and new sampling techniques specific for groundwater has provided scientists with new tools to explore subsurface habitat. Indeed, recent studies have shown evidence for many biotic processes in groundwater, e.g. transforming organic carbon, nitrogen and sulfur; degrading pesticides, fuels and synthetic organic pollutants (Chapelle, 1993). These evidences prove that the groundwater environment can be a habitat for microbial life.

Based on this outcome, one consider that, although it is certainly true that groundwater from deep and confined aquifers is the least susceptible to microbial contamination with respect to the other water sources, there is still the risk (potential) of fecal or general microbial contamination. This is particularly true for unconfined and shallow aquifers.

Some microorganisms (bacteria, protozoa and viruses) that can be found in ground waters may cause disease in humans. These organisms are commonly called pathogens, and are closely associated with humans and other warm-blooded animals, because they are usually transmitted from one organism to another by direct contact, or by contamination of food or water (Tortora, 2004). The mechanism of transmission of these diseases is very simple: cells of the pathogen are shed in the feces of one organism, and if these cells contaminate food or water in sufficient number, which is then consumed by another organism, the disease spreads. Some pathogenic

microorganisms may not be closely associated to humans or other mammals, but they rarely cause diseases in healthy adults. Older people, children, sick and debilitated people, and pregnant women are generally considered sensitive subjects and may be affected by waterborne infections that usually do not affect the rest of the population.

Feces of humans and other warm-blooded animals contain a huge number and variety of microorganisms. Fecal Coliforms and Fecal Streptococci are two groups of bacteria belonging to the human and animal gastrointestinal flora. They are always released into the environment with feces, and are then exposed to a variety of environmental conditions that eventually cause their death. These bacteria are generally not pathogenic. Nevertheless, fecal coliforms and streptococci are relevant because they are the main indicators of fecal pollution in surface and groundwater.

It is assumed that fecal bacteria cannot grow in natural environments, since they are adapted to live in the gastrointestinal tract. Studies have shown that these bacteria survive from a few hours up to several days in surface water, but they might survive for days or months in sediments, where they are protected from sunlight and predation by protozoans, creating a microenvironment where they can regulate the conditions to the optimal level for their survival (Bitton, 1984; Henis, 1987).

There is still controversy of whether fecal bacteria, microbial pathogens and viruses can survive in groundwater. It is commonly believed that low temperature (7 - 12 °C), competition with autochthonous bacteria and

entrapment in soil may all contribute to their demise, or in general slow down their diffusion from a source. Several studies have been conducted in this field: Abu-Ashour (1993; 1994; 1999) published the results on the movement of bacteria through homogeneous and heterogeneous soils; Conboy (2000) tried to identify profile characteristics that make an aquifer susceptible to microbial contamination; Reaume (1993; 1994) and Joy (1998) investigated the dispersion of bacteria in soil as a consequence of field application of liquid manure; Seyfried (1995), Burnham (1995) and Shadford (1993; 1994) did research on the dispersion of bacteria and viruses form septic systems; in particular Burnham (1997), Shadford (1997) and Joy (1994) used a biotracer (Nalidixic acid – resistant *E. coli*) to quantify how far from various septic system can bacteria spread; Jamieson (2002) published a recent review on the movement and persistence of fecal bacteria in agricultural soils; Jin (2000) studied virus sorption and inactivation during transport through sand columns; Nasser (1999) did an interesting study on the environmental factors affecting the inactivation of viruses that may cause waterborne diseases.

According to these studies, bacteria, viruses and other endogenous microorganisms have a limited life in groundwater (Table 2.1), and generally they do not travel significant distances from the source of emission (e.g. 15-20 m from the source for properly designed septic systems) (Bitton, 1984). Nevertheless, there might be particular situations where pathogens and other microorganisms can survive longer and affect targets quite distant from the source, e.g. in case of fractured aquifer, and with particular geochemical

conditions.

Table 2.1 – Infectious agents and parasites of pigs transmissible to humans (adapted from Fairley, 1996)

Type of Infection	Etiological Agent
Bacterial	Leptospira sp. Erisipelothrix rhusiopathiae Campylobacter jejuni Salmonella sp. Mycobacterium sp. Yersina sp. Anthrax
Protozoan	Ascaris suum Toxoplasma gondii Trichinella spiralis Pulex irritans Sarcoptes scabei Balantidium
Viral	Encephalomyocarditis virus
Mycotic	Ringworm

Generally, it is assumed that the die-off of pathogenic microorganisms is similar or faster than that of the fecal indicators under the same conditions. Therefore, if a relatively high number of fecal indicator bacteria is found in a water environment, this means that it is very likely that pathogens are present as well, while their absence should mean absence of pathogens associated to feces. For this reason, it is usually convenient to adopt the determination of a bacterial indicator of fecal contamination rather than the pathogen itself. Unfortunately, some pathogenic microorganisms may have special survival mechanisms, such as cyst formation in *Cryptosporidium*, spore formation in Clostridia, or attachment to particles in viruses, so that waters free of fecal indicator bacteria might still represent a risk for waterborne diseases in some particular situations.

The presence of fecal pollution in an aquifer is always related to a contaminated surface environment, from here on defined as "source". The most common sources of groundwater microbial contamination are: waste lagoons or contaminated surface water, septic tanks, broken or leaking sewer lines, old or improperly designed or leaking landfills, and potentially field spreading of animal manure (Gerba, 1999).

Despite all the recent advances in subsurface microbiology, relatively little is known about microorganisms in groundwater habitat, and even less is known about pathogens and their correlation to waterborne diseases. In fact, the discovery of new pathogenic bacteria or protozoa, associable to specific diseases, still occurs frequently nowadays (Sinton, 1998).

2.2 – Health risks related to fecal contamination in surface and ground waters

According to the Center for Disease Control and Prevention (CDC, 2000), it is estimated that there are from 3 to 5 billion episodes of diarrhea every year in the world, which result in an estimated 3 million deaths, mostly among children. Waterborne bacterial infections may account for as many as half of these episodes and deaths. In a recent report regarding the emerging infectious diseases published by CDC (1994), Cryptosporidiosis and *E. coli* O157:H7 diseases are included among the infections that deserve particular attention and study, and they are both waterborne pathogens. The most common etiologic agents related to waterborne diseases include *Vibrio cholerae*, *Campylobacter*, *Salmonella*, and *Shigella*, and the subjects at risk are certainly the persons living in poverty in the developing world. However, microbial contamination of ground and surface waters is not only a problem of the developing countries. According to Bitton and Gerba (1984), it is well known since the beginning of the '80 that two-thirds of all rural household drinking water in North America were unacceptable from chemical and bacteriological standpoints. Craun (1991) reported that about 44 % of the outbreaks in the United States were associated with contaminated groundwater. Recently CDC (2003) has estimated that as many as 900,000 cases and 900 deaths annually in the USA are due to waterborne diseases. Similar studies, conducted in Canada, have shown that about 40 % of the farm wells are contaminated by fecal bacteria (Goss, 1998).

According to the Canadian National Water Research Institute (NWRI,

2003), between 1974 and 1996, more than 200 infectious disease outbreaks related to drinking water were reported. Concerns about waterborne pathogens and elevated bacteria levels have also adversely affected many recreational waters and shellfish areas across Canada.

Tragically well-know is the outbreak that occurred in Walkerton - Ontario in May 2000. From the inquiry instituted after the contamination, it emerged that the primary organisms responsible for the 2300 gastroenteritis and 7 deaths were *Escherichia coli* O157:H7 and *Campylobacter jejuni*, arising from cattle manure from a local farm after a period of exceptionally heavy spring rainfall. One of the factors that probably contributed to this event was the unfavorable typology of the aquifer in the area of the production wells (Hrudey, 2002; Report on the Walkerton Inquiry, 2001).

The facts so far stated make one assume an unequivocal relationship between the degree of fecal contamination of water sources and the probability of incurring in some form of waterborne infection. However, there is little direct epidemiological evidence proving a correlation between the transmissibility of waterborne diseases and the level of fecal indicators in drinking water. In a recent study, Payment (2000) established a significant correlation between certain fecal indicators (total coliforms, fecal coliforms and *C. perfringens*) and certain waterborne pathogens (*Giardia lamblia*, *Cryptosporidium*, human enteric viruses) in river waters used as drinking water source, but studies of this kind are very sporadic. Most of the epidemiological evidence relating waterborne pathogens to the concentration

of certain fecal indicators is in fact derived from studies regarding recreational water.

There are over 100 different types of bacteria, protozoa and viruses which can be found in contaminated waters. According to Craun (Craun, Chapter 7 in Bitton, 1984), the prevailing infectious diseases that can be transmitted by contaminated drinking water include:

- Amebiasis (micr. agent: *Entamoeba histolytica*, protozoan)
- Balantidiasis (micr. agent: *Ballantidium coli*, protozoan)
- Cholera (micr. agent: *Vibrio cholerae*, bacterium)
- Dracontiasis (micr. agent: *Dracunculus medinensis*, nematode)
- Gastroenteritis: Undetermined etiology
  Campylobacter
  Toxigenic *E. coli*
  Viral
- Giardiasis (micr. agent: *Giardia lamblia*, protozoan)
- Hepatitis (micr. agent: Hepatitis A, virus)
- Paratyphoid Fever
- Salmonellosis
- Shigellosis (micr. agent: *Shigella* sp.)
- Typhoid Fever (micr. agent: *Salmonella typhi*, bacterium)
- Tularemia

Only Dracontiasis is transmitted exclusively through drinking water; the transmission of all the others happens generally through the fecal-oral

route. Some of these infections occur rarely in North America, e.g. cholera, while some are becoming more frequent, e. g. giardiasis, and the list of the concerning pathogens has been recently updated to include the following microorganisms (USGS, 2000):

- *Cryptosporidium parvum*
- *Helicobacter pylori*
- *Tropheryma whippelii*, an actinomycete
- Adenoviruses
- Astroviruses
- Coxsackievirus B
- Rotaviruses
- Norwalk viruses.

There is medical evidence that a large proportion of the waterborne infections are likely to be of viral origin, therefore, there is more concern for the fecal contamination of human origin. Nevertheless, there is an increasing concern for the so called zoonoses (Sinton, 1998; Fairlay, 1996).

By definition, zoonoses are the diseases with animal as reservoirs of infection. Generally, the more similar an animal is to humans (i.e., the more closely related evolutionarily), the more likely the animal will be able to serve as a reservoir for a human disease, as well, of course, to be susceptible to human diseases. A complete list of the emerging zoonoses is given by CDC (2003). Some of the pathogens listed above should be included among infective zoonotic microorganisms, e.g. *Giardia* sp..

Nowadays, the World Heath Organization (2001) is more concerned for foot-borne zoonoses (e.g. Mad Cow Disease) and similarly are all the other regional and local health and veterinary organizations and institutes. Fairley (1996) conducted an interesting study that investigated the zoonoses associated with pigs (Table 2.1).

From these facts, the importance of distinguishing human from animal fecal contamination is evident. The identification of the source helps from one side the water management activities, and from the other it is indispensable information to develop epidemiological evidence of the transmission of waterborne diseases, and therefore to increase the understanding of waterborne pathogens.

2.3 – Persistence of pathogens and other microbes in groundwater

The persistence of pathogens in groundwater is one of the most important factors affecting the health risk. Because waterborne pathogens can be protozoa, bacteria or viruses, it is convenient to refer to their inactivation as a parameter to quantify their persistence in a habitat (Bitton, 1984). Therefore, the persistence is not measured strictly as survival of the individual microbes, but as the ability of reproduction in bacteria and protozoa populations, and the overall infectivity of populations of viruses. According to this definition, there still can be, for example, inactivated but viable cells, able to nourish and to accomplish other functions for themselves, but unable to reproduce (Henis, 1987). Moreover, inactivation is defined as an irreversible process; therefore, if for example, a microbe loses its ability to reproduce (or to infect a host cell, in case of virus) it will not recover this ability even if moved to a favorable habitat. In general, one may say that a pathogenic microorganism is inactivated when it is becomes permanently unable to infect.

Many factors affect the inactivation of microorganisms, depending mainly on the habitat where the microbe lives (Bitton, 1986). In case of waterborne pathogens in groundwater, these are:

- environmental conditions (temperature, pH, water chemistry, soil characteristics);
- biotic conditions (presence of predators, competition with other microorganisms);

- genetic characteristics of the population; and
- history of the population (e.g. whether the population has grown in rich or poor nutrient medium, etc...).

In case of bacteria or protozoa, three different patterns, shown in Figure 2.1, have been identified for the decline of the population (Henis, 1987). These are represented by three different curves:

- Sigmoid;
- Exponential;
- Exponential preceded by a lag phase.

If it is assume that there is a single event that leads to inactivation, the rate of inactivation will depend on the concentration of cells at every moment.

This can be represented by the equation:

$$\frac{dX}{dt} = -kX \qquad (1)$$

Where X is the bacterial concentration [#cells/L], t is the time [d] and k is a constant [d^{-1}] that depends mainly on organism hereditary background, history and environmental conditions (Henis, 1987). Thus, under ideal conditions, the concentration of bacterial fits in a straight line when plotted on a semi-logarithmic scale. Deviations from this ideal situation are brought about by unevenness in the population, e.g. different history, or different susceptibility to the environmental conditions inside the same population.

Xiong and coworkers (1999) described six different equations for the inactivation of bacteria, shown in Figure 2.2. Each single equation can describe with more accuracy the specific behavior of certain species or it can follow the behavior of the same species in different environmental conditions.

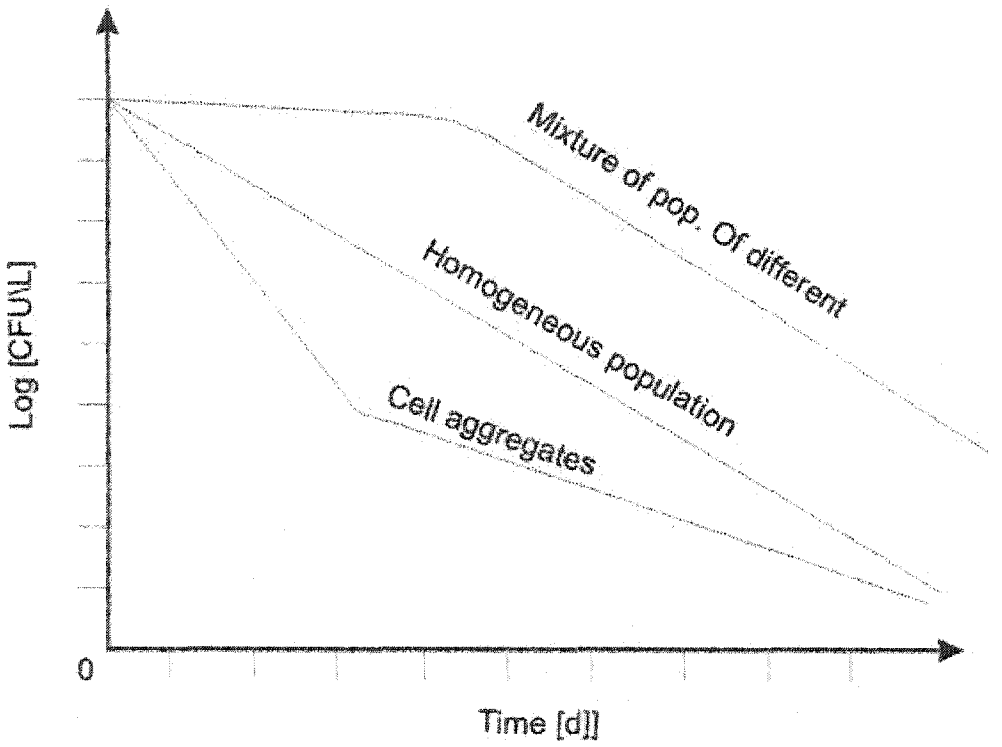


Figure 2.1 – Representation of different shapes of inactivation curves for bacteria (adapted from Henis, 1987)

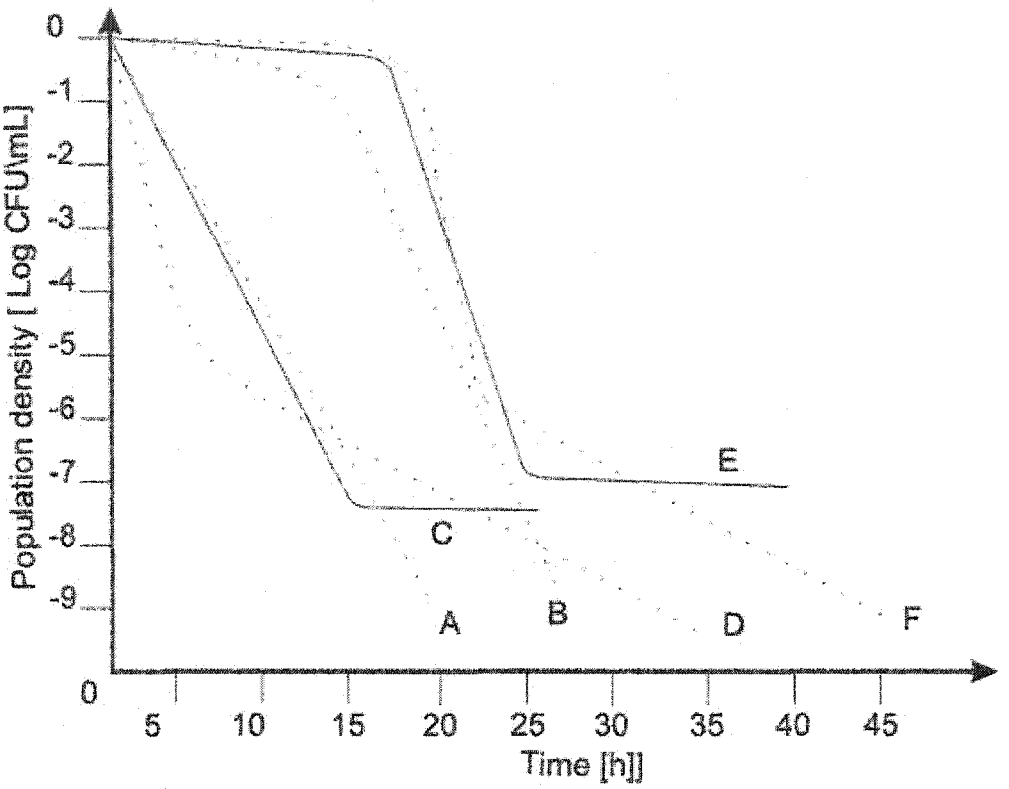


Figure 2.2 – Other shapes of inactivation curves for bacteria (adapted from Xiong, 1999); A is a linear type of survival curve, B is a curve with shoulder, C and D are curves with a tailing (also called biphasic), E and F are sigmoidal curves.

Enteric bacteria have a very limited capacity to multiply in alien environments. Studies conducted on the behavior of this group of bacteria in surface and ground fresh waters, as well as in marine environments revealed different rates of disappearance depending on the different temperature, salinity, aeration, pH, Eh, predatory protozoa and lower metazoans, attack by bacteriophage and limited competition capacity for nutrients (Henis, 1987). It

appears that nutrients and temperature play an important role, the former increasing the survival with increasing levels of nutrients, while the latter decreasing the survival at higher temperatures. The average survival for certain species in groundwater is reported in Table 2.2 (Henis, 1987).

Viruses, because of their peculiar characteristics, deserve a few distinctive notes. Because they are intracellular parasites that replicate only within their specific host cells, their inactivation can be obtained only by the partial or total destruction of their structure or by damage of at least one of the structural parts (polypeptides) involved in the infective mechanism. Usually environmental factors affect the protein coat, the genetic material, or both. The factors that may influence enterovirus survival in water and soil are (Gerba, 1999):

- Physical factors:
  - Temperature — longer survival at lower temperatures
  - Radiation — influence only on soil surface
  - Desiccation — fast inactivation in dry soils
- Chemical factors:
  - pH — influences the adsorption to soil
  - Cations — increase the adsorption to soil
- Biological factors:
  - Bacteria, algae — may reduce persistence in water
  - Extracellular Bacterial products — involved in the inactivation in water.

Table 2.2 – Die-off rate constants for certain pathogens and other relevant microbes (Adapted from: Gerba, 1999; Medema, 1997; Bitton, 1984).

Microorganism	Die-off Rate [Log10/day]
Poliovirus I[i]	0.046 0.21 0.77
Coxsackievirus[i]	0.19
Rotavirus SA-11[i]	0.36
Coliphage T7[i]	0.15
Coliphage f2[i]	1.42 0.39
Escherichia coli[i]	0.32 0.36 0.16
Fecal Streptococci[i]	0.23 0.24 0.03
Salmonella typhimurium[i]	0.13 0.22
Cryptosporidium (excystation)[ii]	0.010
Cryptosporidium (dye exclusion)[ii]	0.010
Enterococcus faecium[ii]	0.077
Clostridium perfringens[ii]	0.003

i) in groundwater
ii) in natural surface waters at 5 °C.

Many studies have revealed that adsorption to soil surfaces may increase significantly the persistence of viruses (Gerba, 1999). It has also been demonstrated that certain bacteria have important antiviral properties (Bitton in Henis, 1987).

The Die-off Rate constants for some relevant pathogens and fecal indicators are shown in Table 2.2. According to Metcalf and Eddy (2003) the average survival times in soil at 20-30 °C is:

- bacteria < 120 days but usually < 50
- protozoa < 20 days but usually < 10
- helminths many months
- viruses < 100 days but usually < 20.

2.4 – Overview of the National Standards for Microbial Water Quality

The Canadian government enforces the water quality standards for microbiological parameters through regulations and guidelines. The levels of tolerance to certain microbiological parameters vary according to the destination and final use of the water resources (Canadian Water Quality Guidelines, 2003). For this reason, the Canadian water quality guidelines have been subdivided into six different categories. In this study, only the standards proposed for drinking, agricultural and recreational water uses are considered. Although only the first two should be related to groundwater sources, recreational water quality standards are also considered, because most of this epidemiological evidence for drinking water is derived from studies related to recreational waters, and because these values are useful information that can be compared to a given level of microbial contamination.

Table 2.3 presents the parameters and the values referred to each water use suggested by Environment Canada.

Table 2.3 - Water quality standards for microbiological parameters in Canada (Adapted from Canadian Water Quality Guidelines, 2003)

Category	Parameter	Maximum Acceptable Concentration (MAC)
Drinking waters	Coliforms, Total	Not detectable per 100 mL in any sample of private drinking water supply system
	E. coli or other fecal coliforms	Not detectable per 100 mL in any sample of public or private drinking water supply system
	Protozoa (e.g. *Giardia* and *Cryptosporidium)*	No MAC proposed but it is suggested monitoring in case of risk (under review)
	Virus	No MAC proposed but it is suggested monitoring in case of risk (under review)
Agricultural waters	Total Coliforms	1000 CFU per 100 mL of sample
	E. coli or other fecal coliforms[i]	100 CFU per 100 mL of sample
Recreational waters	Coliforms, fecal	Less than 2000 CFU per L in 5 samples (+ re-sampling)
	Coliphages	Not specified
	Enterococci	Less than 350 CFU per L in marine water samples
	Pathogens, bacteria	No limits are specified, but it is suggested the use of the following species as a parameter to assist in interpreting the results of sanitary and microbiological survey: *Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella, Shigella, Aeromonas, Campylobacter jejuni, Legionella*
Continue to next page		

CFU = Colony Forming Unit.

[i] Fecal coliforms are here referred as thermo-tolerant coliforms.

	Pathogens, viruses	No limits are specified, but it is suggested the use of the following species as a parameter to assist in interpreting the results of sanitary and microbiological survey: enteroviruses (polio, coxsackie, echo, and hepatitis A viruses), adenoviruses, rotaviruses, reoviruses, Norwalk viruses, caliciviruses, astroviruses, and coronaviruses
	Pathogens, protozoa	No limits are specified, but it is suggested the use of the following species as a parameter to assist in interpreting the results of sanitary and microbiological survey: *Giardia, Cryptosporidium, Naegleria, and Entamoeba histolytica*

Heath Canada recently established a guideline specific for viruses in drinking water (Health Canada, 2003). The agency focuses its attention on human enteric viruses, because of their host-specificity. According to AWWA more than 140 different serological types of human enteric viruses have been described (Epa, 1993), and those associated with waterborne illness include: Hepatitis A virus, Hepatitis E virus, Norwalk and Norwalk-like viruses, Rotaviruses and enteroviruses (polioviruses, coxsackieviruses A and B, echoviruses and 4 ungrouped enteroviruses). In protected groundwater with temperatures generally below 10°C, enteric viruses have been reported to survive for nearly 2 years. Therefore, it is advised to use specific treatment technologies that can achieve at least a 4-log reduction and/or inactivation of viruses before using water for drinking purposes.

Water quality standards proposed in other countries of the world are

quite similar to Canadian guidelines. In the USA and Europe, the governments are involved through the environmental agencies in the study of new and improved methods for the measurement of microbial quality of waters. It is likely that in the next few years new parameters and methods will be proposed as drinking water quality indices (US EPA, 2003; EU, 2003).

2.5 – Overview of the Microbial Source Tracking Methods

As briefly stated in the introductory paragraphs, the direct detection of waterborne pathogens is not always feasible for the following reasons:

- There is only a partial understanding of the existing pathogens and new infective species continue to be discovered
- The direct measurement of pathogens is often difficult and technically challenging, because they are present only in small numbers and because the methods are not enough sensitive and developed
- The direct detection is often time-demanding and very expensive.

Therefore, it is still convenient (and safe) to infer the presence of pathogen microorganisms through an indirect measurement, and the most consolidated way is to do it through the determination/enumeration of a fecal INDICATOR. In order to obtain reliable information, the chosen indicator should match certain criteria.

The characteristics that an ideal fecal indicator should generally have are (Maier, 2000):

1. The indicator must be present whenever fecal contamination is present (in case of a microorganism, it should be a member of the microflora of warm-blooded animals; in case of other substances it should be associated solely to fecal discharge).
2. The indicator should not be present in the environment other than when there is fecal contamination (in case of a microorganism, it should not grow in the environment).

3. The indicator should be good for all types of environment (surface, marine and ground waters).
4. The concentration (number) of indicator(s) present should be greater or at least equal to that of the pathogen.
5. The indicator must have a reasonably longer "survival time" (persistence) than the hardiest pathogen for which it is a surrogate.
6. The quantification of the indicator (sampling and measurement included) should be faster, easier to perform and more sensitive than that of the target pathogen.
7. The quantification of the indicator (sampling and measurement included) should be cheaper than that of the target pathogen.

These characteristics have been identified with the purpose of assessing fecal contamination in a water environment, and with the assumption that fecal contamination is directly relate to the presence of pathogens (adapted from Metcalf & Eddy, 2003). It should be noted that these criteria can be applied to chemicals as well as to microbial organisms.

Besides the need to detect the presence of fecal contamination (and pathogens), the identification of the sources of fecal contamination is also becoming important. As previously specified, the most common sources of groundwater fecal contamination are:

- Waste lagoons (domestic animal source)
- Contaminated surface water (wild or domestic animal source)
- Septic tanks (human source)

- Broken or leaking sewer lines (human source or domestic animal when there is a sewer system in the farm)
- Spreading of manure (domestic animal source).

The identification of the sources (human, domestic animal, wild animal or other) can be of great help to water managers, e.g. for the reclamation of a contaminated aquifer, or for the development of Total Daily Loads in specific watersheds, and above all can increase the understanding of contamination through waterborne pathogens.

Sometimes there are sufficient direct or indirect information that can be used to identify the source of pollution. These are:

- Geographical information of the surroundings of the contaminated water body, including location and characteristics of potential sources
- Geologic and hydrogeologic data of the surroundings
- Historical record of microbiological water quality for the contaminated water body
- Direct visual evidence like, for example, manure spreading on the fields.

Most of the time, this information is not enough or is controversial, and it is necessary to "track back" the source of pollution from the point of contamination using other specific methods.

The techniques, indeed applicable to distinguish pollution sources both in ground and surface waters, are commonly called Microbial Source Tracking

(MST) methods or Bacteria Source Tracking (BST) methods.

The criteria specified above for the ideal fecal indicator can be partly adapted to outline the characteristics of the Ideal Microbial Source Indicator, but since in this case the identification of a fecal source (mostly human or animal) is more relevant, these new criteria will have characteristics more similar to those applied to groundwater tracers (Gerba, in Bitton, 1984; Chapelle, 1993).

The characteristics that an ideal MST Indicator should generally have are:

1. The presence of the indicator can be related only to one source (human or animal, but it can be also more restrictive, e.g. it can be related only to pig fecal contamination from pig or cattle, etc...).
2. The indicator must be detected in a particular environment whenever there is fecal contamination of the specific source that it is supposed to identify.
3. The indicator should satisfy other criteria of the ideal fecal indicator stated at the beginning of the paragraph.

In this case, a longer persistence of the indicator in the environment is desired because it can serve to track fecal contaminations occurred in the past even when the usual indicators are no more detectable. Indeed, the use of indicators having different measurable persistence in a specific environment can be used for timing the pollution event. It should be noted that the criteria for the ideal MST indicator can be applied both to a chemical

substance and to microorganisms.

There are only two comprehensive reviews on the MST methods: the more recent was published by Scott (2002), while the other was by Sinton (1998). According to these studies, MST methods can be classified as follows:

I. Microbiological methods

   a. Fecal Streptococci, subdivided in:

      - Fecal Coliforms / Fecal Streptococci (FC/FS)
      - Fecal Streptococci species identification
      - FC/FS shift

   b. Bifidobacteria spp., in particular Sorbitol fermenting Bifidobacteria

   c. *Rhodococcus coprophilus*

   d. Bacteroides spp.

   e. Phages of *Bacteroides fragilis*

   f. F-RNA phage subgroup

   g. Eggs of helminthes (intestinal worms)

   h. *Pseudomonas aeruginosa*

   i. Human enteric viruses

II. Phenotypic methods

   a. Antibiotic Resistance Analysis (ARA) and Multiple Antibiotics Resistance Analysis (MAR)

   b. Serogrouping

III. Genetic methods

   a. Ribotyping

   b. Host-specific molecular markers

c. Pulse-field gel electrophoresis (PFGE)

IV. Chemical methods

a. Fecal Sterols

b. Fluorescent whitening agents

c. Sodium tripolyphosphate

d. Long-chain alkylbenzenes

e. Caffeine

f. Aspirin

g. Estrogens

h. Antibiotics (for detection of livestock fecal pollution)

i. Other Drugs and Personal Care Products.

2.5.1 – Microbiological methods

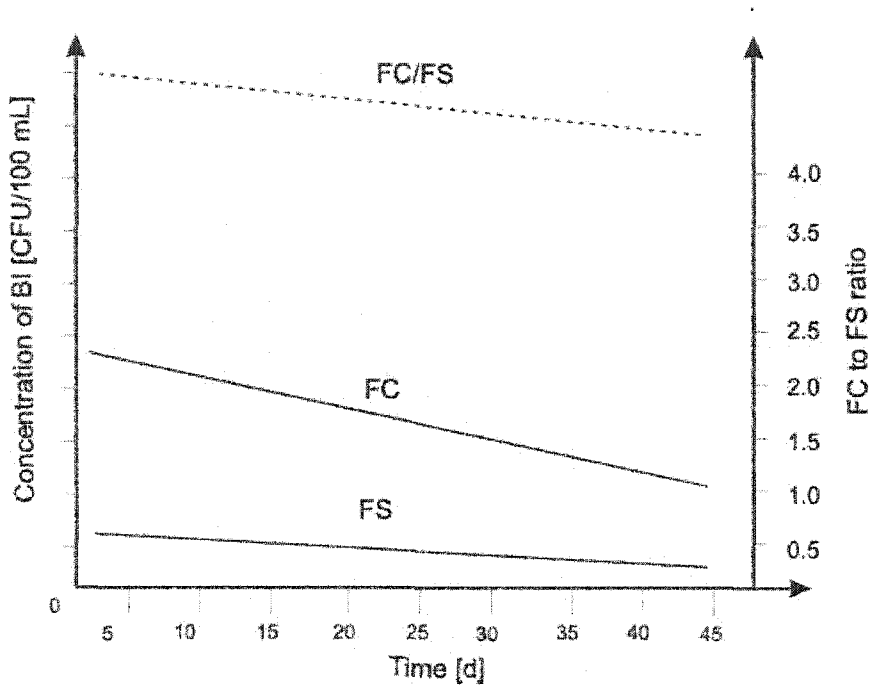
These methods are based on the isolation of a specific microorganism (or group of microorganisms) from all the other species polluting a water body. This microorganism should be exclusively a member of the microflora of one of the suspected sources (human or animal). In the case of bacteria, the isolation is usually obtained by culturing a dilution of the sample (but most often bacteria are attached to a filter) in a selective medium. Sometimes the procedure comprises other steps to confirm the identity of the presumptive isolate. When the indicator is a protozoan or virus, specific microbial isolation methods are used.

The drawback of these methods is that there are only a few organisms that are host specific, and they are not always easy to isolate.

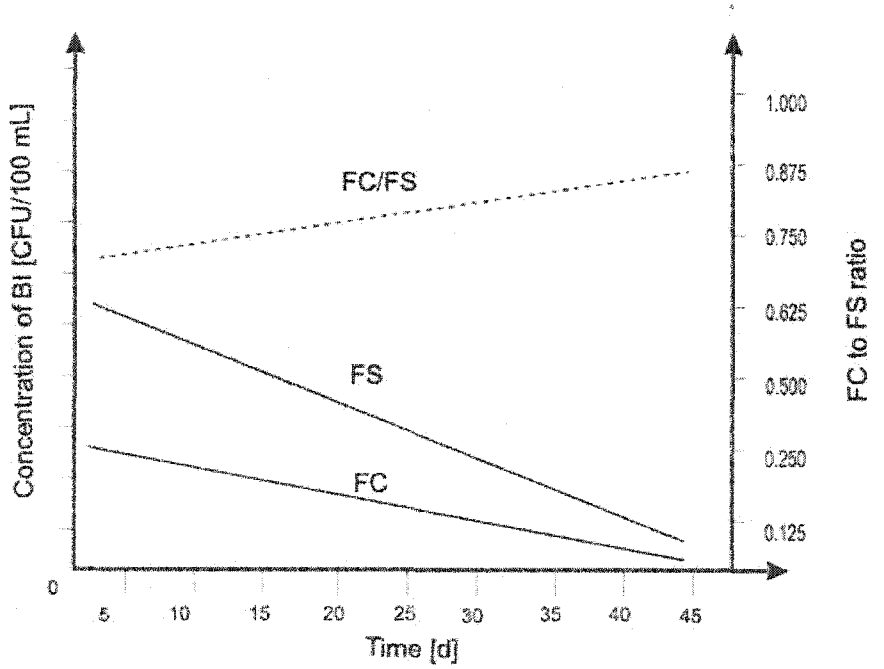
a) Fecal Streptococci

The group of Fecal Streptococci is considered to comprise the enterococci species (*Enterococcus faecium, E. faecalis, E. durans, E. avium, E. gallinarum*) together with two non-enterococci (*Streptococcus equinus, S. bovis*). The enumeration method is membrane filtration, in conjunction with m-Enterococcus or KF agar as a growth medium. The method is described in Sec. 3.1.

Fecal Streptococci have been studied extensively in the past, and most studies have revealed that they are more persistent than fecal coliforms in groundwater (Geldreich, 1976).



(a)



(b)

Figure 2.3 – FC to FS ratios and the ratio shift in human (a) and animal (b) feces versus time.

Three different methods have been proposed for Fecal Streptococci as indicators of fecal contamination sources:

1. Fecal Coliforms vs. Fecal Streptococci Ratio (FC/FS): according to Geldreich (1969), a ratio greater than 4 indicates human feces (FC/FS > 4), while if it is less than 0.7 indicates animal feces (FC/FS < 0.7).
2. Species Identification: this method is based on the different ratio of enterococci and streptococci species in feces statistically determined for different warm-blood animals, according to which human feces contain predominantly enterococci species, while animal feces have a significant number of non-enterococci. In this case, the use of a specific growth medium for non-enterococci is required. The total number of Fecal Streptococci is determined using the media already mentioned (see Figure 2.3).
3. Ratio Shift: this approach is based on the interpretation of the differential die-off of fecal coliforms and fecal streptococci in stored samples. Human source, dominated by enterococci, which are more persistent than FC, should exhibit initially an higher FC to FS ratio (> 4) which should than decrease with time, while non-human sources, dominated by *S. bovis* and *S. equinus*, that are less persistent than FC, should have an initially lower FC to FS ratio, which should then rise.

These methods, although rapid in giving results (except the ratio shift) and requiring minimal expertise, have proven to be unreliable and have therefore been abandoned or advised against.

b) Bifidobacteria Species

Bifidobacteria is a group of microbes that are present in very high numbers in human feces, in particular *B. adolescentis* and *B. longum*. Some species have been found also in animals but never in unpolluted environments. Some of the Bifidobacteria hosted exclusively by humans have the ability to ferment Sorbitol; this subgroup, called Sorbitol Fermenting Bifidobacteria (SFB), is composed of *B. adolescentis* and *B. breve*, and can be used as indicator of human source of fecal pollution.

Presently, only one growth medium has been formulated for the isolation of SFB, the Human Bifidobacteria Sorbitol-fermenting Agar (HBSA). This medium can be used in conjunction with membrane filtration (method described in Sec. 3.1) (Mara, et al., 1983), but, according to recent studies, there is still the need to improve the selectivity and sensitivity of this method.

In the last few years, new studies have been published on the use of Bifidobacteria species as indicators of human fecal contamination, renewing the interest for this group of microorganisms in relation to MST methods. Lynch and coworkers (2002) used the Bifidobacterium medium (BFM), recently developed by Nebra (1999), for the recovery and isolation of Bifidobacteria from the water samples (sewage effluents); after isolating the colonies. They identified *B. adolescentis* (human specific) by using colony hybridization with a digoxigenin (DIG-)-labeled oligonucleotide probe. Just before the end of this study, Nerba and coworkers (2003) published a paper

on the use of *B. dentium* (human specific) as an indicator of the origin of fecal water pollution. In this case *B. dentium* was detected using 16S rRNA gene-targeted probes. Because of the intensive use of genetic techniques, these new MST methods are not to be considered purely microbiological. The distribution of Bifidobacteria in different environments has been described by Ventura et al. (2001) and by Gavini (BIFID-Project, 2003).

c) *Rhodococcus coprophilus*

R. coprophilus is an actionmycete that can be found in herbivores dung and pasture run-off, but it is absent in human feces. For this reason it can be used as a specific indicator of grazing animals' fecal contamination. Its persistence in waters and sediments is considerably longer than that of the FS and other fecal indicators. The method for recovery and enumeration of *R. coprophilus* is complex. Before plating, interfering bacteria are reduced by heat treatment of the samples at 55 °C for 6 minutes; it is followed by incubation of the plate using M3 modified agar at 30 °C for 12-14 days; and the last step involves the exposure of the plate to sunlight (500-1500 lux intensity) for 4-7 days to enhance the pigmentation of the colonies (Oragui, 1983).

R. coprophilus can be a reliable indicator of domestic herbivores' fecal contamination, but because of its persistence it cannot distinguish remote from recent contamination and, therefore, it is suggested its use in combination with some other fecal indicator.

d) Bacteroides Species

Bacteroides are among the most numerous bacteria in human feces, 100 times greater in number than *E.coli*, while these are almost absent in animals' feces; hence has potential role as indicator of human sources of fecal contamination. Among all the species know, *B. fragilis* has been found only in human feces at very high numbers. Only a few methods for recovery and enumeration of Bacteroides species have been studied. The most common way to isolate *B. fragilis* is by use of Bacteroides Bile Esculin Agar (BBE) (Livingston et al., 1978). Another method involves the use of WCPG medium, which can be used in conjunction with membrane filtration, and should allow the isolation of Bacteroides species (Wilkings et al., 1976). However, this method is still under development and is time demanding. According to Sinton (1998), the development of a reliable method for isolating, identifying and enumerating Bacteroides species will depend on DNA-based techniques.

e) Phages of *Bacteroides fragilis*

As stated in the previous section, some Bacteroides species are host-specific. In particular, *B. fragilis* has been found only in human feces and could be used as human specific indicator. Although there are not many studies on the direct use of *B. fragilis* as fecal source indicator, there are evidences that its persistence in the environment is low.

Tartera and his coworkers (1987) had the idea of using a Bacteriophage, which is a virus that infects bacteria, as a human specific

fecal indicator. This Bacteriophage is specific of *B. fragilis* and significantly more persistent than its host in water.

The methods of enumeration of Bacteriophages of *B. fragilis* are the double-layer agar technique (with plaque detection), using Bacteroides Phage Recovery medium (BPRM). Although this technique is not very complex (Pepper, 1995), and Bacteriophage of *B. fragilis* are very specific indicators for humans, there are still perplexities about the use of this method, mainly because it has been observed that, sometimes in waters polluted by human feces, these phages are not detected (Sinton, 1998).

f) F-RNA phage subgroup

F-RNA phages are a group of icosahedral phages that attach specifically to the F-pili of bacteria (filamentous structures on the cell walls of "m" bacterial strains). F-RNA coliphages infect coliform bacteria; in particular there are two subgroups (II and III) that have been isolated only in human feces, while subgroup I has been found only in non-human mammals (Sinton, 1998). The methods of detection are various and once detected, the coliphages subgroups can be identified using immunological or genetic tests.

These methods have not been comprehensively tested yet, but it appears that they are time consuming and expensive (Sinton, 1998).

g) Others

Because enteroviruses possess a high degree of host-specificity, it is certainly true that their detection in water is a clear index of human fecal

contamination. Furthermore, they give most direct information to assess the risk brought about by waterborne pathogens. Nonetheless, waters polluted by human feces do not necessarily contain enterovirus; therefore, enteroviruses are not always reliable indicators of the source of contamination. The methods of detection of these pathogens, based on PCR techniques, are reviewed by Toze (1999). It should also be remembered that these methods are cumbersome and expensive, as already mentioned in the introduction. The eggs of helminths (intestinal worms, host-specific) are another method under investigation, but there is still not enough evidence to evaluate it. On the other hand, *Pseudomonas aeruginosa* has been used as a source specific indicator, but many shortcomings are present, including rapid die-off in the environment, possible growth outside warm-blooded animals, and presence in plants and soils; therefore, its use as a source indicator has been abandoned (Scott, 2002).

h) About the use of *Clostridium perfringens* as a source indicator

Scott and coworkers (2002) included *Clostridium perfringens* among the indicators for water quality, considering it particularly useful in specific situations, like for example to detect remote fecal pollution or to evaluate the efficiency of disinfection processes in water and wastewater treatment plants. In addition to that, they indicated the presence of *C. perfringens* both in human and in animal feces, excluding any claim of using it as indicator of sources of fecal pollution.

The same point of view is assumed by US-EPA (EPA/600/R-95/178, US

EPA, 1996), Bisson and Cabelli (1979), who developed the mCP medium (see appendix) and the membrane filtration procedure for waters (Payment, 1993; Sørensen, 1989).

There is a study by Conboy and Goss (2001) where the presence of *C. perfringens* was related uniquely to animal feces. In particular, the Shahidi-Ferguson Perfringens agar (Shahidi, 1971), which is usually applied for the detection of *C. perfringens* in food samples, was used in screw cap test-tubes (presence/absence test) for the detection of *C. perfringens* in groundwater samples and in dilutions of manure samples. The researchers tested many samples of animal feces, for many different species, together with samples of waters from septic systems, and they always obtained positive results (presence) except in the septic system waters (absence). From these results, the authors concluded that *C. perfringens* can be probably used as a reliable indicator of contamination from animal sources as opposed to septic system in groundwater.

Table 2.4 summarizes the characteristics of the most important organisms used as indicators of human or animal sources of fecal contamination.

Table 2.4 – Characteristics of the most important microbes used as source indicators (Adapted from Mair et al., 2000 and Krieg et al., 1984)

Fecal Coliforms	Fecal coliform is a group of bacteria that includes members of the family *Enterobacteriacae*, which include *Escherichia coli , Citrobacter*, *Enterobacter* and *Klebsiella* species. These gram negative bacilli (rod shaped bacteria) are found in the digestive tracts of all warm-blooded animals. They can be differentiated by their ability to ferment lactose with the production of acid and gas at 44.5 °C within 24 h.
Fecal Streptococci	Fecal Streptococci are a group of gram-positive Lancefield group D streptococci. They belong to the group *Enterococcus* and *Streptococcus*. The members of the Enterococcus genus are different from other streptococci by their ability to grow in 6.5 % sodium chloride, pH 9.6 and 45 °C, and they include: *E. avium, E faecium, E. durans, E. facculis* and *E gallinarium*. Of the genus Streptococcus, only *S. bovis* and *S. equinus* are considered to be Fecal Streptococci.
Bifidobacteria	The genus *Bifidobacterium* is the third most numerous bacterial population in the human intestine after the genera *Bacteroide* and *Eubacterium*. They can also occur in various animals' intestine and in honey bees. Bifidobacteria are rods of varying shape: short, regular, long cells bended, slightly bifurcated. They are gram-positive, non spore-forming, non motile and anaerobic. The species found only in human feces are: *B. adolescentis, B. angulatum, B. bifidum, B. breve, B. catenulatum, B. dentium, B. gallicum, B. infantis, B. longum, B. pseudocatenulatum*. Some of the species that are found only in animals are: *B. animalis, B. boum, B. choerinum, B. gallinarum, B. pseudolongum, B. pullorum* and *B. suis*. Two species can ferment Sorbitol, *B. adolescentis* and *B. breve*, and they are present only in human feces.
	Continued to the next page

Clostridium perfringens	*C. perfringens* is a sulfite-reducing anaerobic bacterium that can form spores. It is rod shaped, Gram-positive. It can produce a number of soluble substances, and based on this quality it has been divided into five types. C. perfringens is a common inhabitant of the gastrointestinal tract of both humans and animals.
Bacteroides fragilis	An obligate anaerobe, *Bacteroides fragilis* is usually a commensal organism, forming a large component of the normal human gut microbiota. However it is also an important opportunistic pathogen. Thirty to fifty percent of fecal matter is said to be *B. fragilis*. It is an obligate anaerobic Gram-negative microorganism, a bit smaller than *E coli*, the ends of the bacilli are rounded. It does not form spores.
Rhodococcus coprophilus	*R. coprophilus* is a natural inhabitant of the feces of herbivores animals, hence its name. It has been isolated from the dung of cows, donkeys, goats, horses and sheep. It is common on grass and in the soil beneath grazed pastures, and is washed into streams and lakes where it can accumulate in the sediment. *R. coprophilus* is a Gram-positive, aerobic actinomycete that forms fungus-like mycelium and breaks up into bacteria-like elements.

2.5.2 – Phenotypic methods

These methods are based on the detection of specific phenotypic characteristics developed by different lineages of bacteria both in animal or human hosts. These phenotypic differences are cause by the different conditions to which the microbes are exposed in the specific host. The drawback of these methods is that often different enteric microorganisms exhibit similar biochemical responses to their environment (Sinton, 1998).

a) Antibiotic Resistance Analysis (ARA)

This method is used to differentiate bacteria having different origin by their different response to antibiotic treatment. In fact, the diet and the antibiotics commonly used in the therapy of humans are different from those used for domestic animals. Therefore, the bacterial flora present in the human intestine will be exposed to a different environment with respect to that typical of domestic animals. This characteristic adaptation profile can be used as a fingerprint to identify the presence of bacteria once they are released in the environment.

The procedure is simple but time demanding. It involves the isolation and culturing of the target organism, then replica plating of the isolates on media containing antibiotic at different concentration. The plated colonies are then observed after incubation and the susceptibilities are recorded for each antibiotic to generate an "antibiotic resistance profile", which is often typical for the strain of the target bacteria adapted to a particular environment (Whitlock, 2002).

ARA has been successfully used in many studies. Huysman et al. (1993) investigated the use of sulphite-reducing Clostridia resistant to tetracycline and oleandomycin as indicators of pig manuring practices. This study was based on the research conducted by Rood and coworkers (1978) on the isolation of multiple antibiotic resistant strains of *Clostridium perfringens* in porcine feces. Whitlock (2002), Wiggins (1999), Harwood (2000) and Graves (2002) used ARA successfully to distinguish the sources of fecal pollution in various environments.

The shortcomings of ARA are the need of a vast database of typical profiles to compare the patterns of the unknown samples, and the potential risk of transferring resistance form one strain to the other. There is still need for more research on this technique.

b) Serogrouping

This method is based on the presence of different somatic (O) antigenic determinants in bacterial strains of the same species. This method has been successfully used in a set of samples coming from different fecal sources. A good percentage has been successfully typed with an insignificant overlapping between the predominant serotypes (Parveen, et al., 2001). Besides the need for more research, this method has the inherent drawback of necessitating a large bank of antisera in order to allow the differentiation.

2.5.3 – Genetic methods

These methods are based on the analysis of DNA sequences of bacteria that can be hosted both in humans or animals, but have developed adaptation to a particular environment or host. The assumption is that the adaptation corresponds to a modification of the genetic code of these individuals, and the genes are transmitted to the progeny. The generic fingerprint of these microbes can be then compared to that of other organisms and to a databank of sequences previously developed (Scott, 2002).

The drawback of these methods is that it is necessary to develop a vast databank, and that there might be inter-species gene transfer between bacteria hosted exclusively in humans or animals. Nonetheless, there are high expectations for these methods, and they are considered to be the most promising MTS techniques for the future (Scott, 2002).

a) Ribotyping

As described by Scott (2002), Ribotyping is a method of DNA fingerprinting that implies the identification of highly conserved rRNA genes (RNA is a structural and functional component of ribosomes) using oligonucleotide probes after treatment of genomic DNA with restriction endonucleases (enzyme that splits DNA or RNA molecule). The method involves the following steps:

- Bacteriological culture and identification
- DNA extraction

- Separation by gel electrophoresis
- Blotting
- Discriminant analysis of the resulting DNA fingerprints.

The Ribotyping technique has been successfully applied by several researchers. Carson and coworkers (2001) used rybotiping on *E.coli* from animal and human feces. Parveen (1999) obtained an average rate of correct classification of about 85 % using this method.

Other fingerprinting methodologies can be applied. Nevertheless, the results of this procedure depend strongly on the availability of an adequate database of profiles to which the fingerprint should be compared. Another shortcoming is that these methodologies are very labor-intensive.

b) Others

Pulse-field gel electrophoresis (PFGE) is another DNA fingerprinting method that involves the analysis of genomic bacterial DNA sequences with rare-cutting restriction endonuclease. This method has been used rarely for fecal source identification (Scott, 2002).

Repetitive element PCR uses primers corresponding to interspersed repetitive DNA elements present in the genome of prokaryotic cells to generate highly specific DNA fingerprints. These molecular technologies applied for the detection of fecal sources have been described by Field (2003).

Once again, the applicability of these methods is limited by the

availability of an adequate databank of profiles.

2.5.4 – Chemical methods

These methods are based on the detection of a chemical or biochemical substance which is not identifiable in unpolluted waters and can be associated only with a specific fecal source. Some of these substances are associated directly with feces, while others are associated simply with fecal discharges.

The drawback of these methods is that they do not give any information on the level of fecal contamination. Therefore, they must be used in combination with some other indicator, and they can also be associated with industrial contamination which does not necessarily represent a risk of infection for humans (no pathogens are present, but probably other noxious substances are present).

a) Fecal Sterols

Many substances are produced by warm-blooded digestion that can be detected in human feces, but out of this set of products only sterols have been successfully used as fecal indicators (Sinton, 1998). The measurement of ammonia nitrogen (NH_3-N) is not a good index of fecal contamination because ammonia can be also the product of rotting vegetation, and is frequently found in unpolluted waters. On the other hand, human metabolites like uric acid and urobilin appeared to be not specific enough (Sinton, 1998).

Fecal Sterols are a group of cholestane-based sterols found in feces.

They comprise coprostanol, sitosterol and campestanol. Coprostanol is produced exclusively in superior mammal species by bacterial degradation of cholesterol, and is the principal human fecal sterol. Therefore, coprostanol can be used as a reliable tracer of fecal pollution. The method of detection is high resolution gas chromatography and mass spectrometry (Nichols, 1993). Unfortunately, coprostanol is also the principal fecal sterol in pigs. Therefore, this method cannot be used to distinguish human from porcine fecal contamination.

b) Fluorescent whitening agents, Sodium tripolyphosphate and long-chain alkylbenzenes

Chemicals contained in liquid detergents and washing powders are usually associated with discharges containing fecal material. Three main substances (or groups of substances) have been investigated as fecal indicators: fluorescent whitening agents (FWAs), sodium tripolyphoshate (STPs) and long-chain alkylbenzenes (LBAs) (Sinton, 1998). The first group of compounds (FWAs) is incorporated in laundry washing powder and can be easily detected using fluorometric measurements (Close, 1989) or thin layer chromatography. The second group (STPs) is a major component of washing powders and can be measured using ion-exchange followed by colorimetric determination. The last one (LBAs) is a group of synthetic hydrocarbons intensively used as anionic surfactants in detergents, and their determination can be done using organic solvent extraction followed by extracts purification using silica gel chromatography.

These substances can also be released with industrial wastewaters,

and may not be strictly related to fecal pollution. This aspect can diminish their usefulness as indicators of human source fecal contamination, especially in case of surface water, but it is likely that there is still a close association to fecal pollution in the case of groundwater in rural areas.

c) Caffeine, Aspirin, Estrogens and other drugs and Personal Care Products

The awareness of the potential risk brought about by pharmaceuticals in the environment started growing in the middle 1990's, when scientists observed deleterious effects on fish and other freshwater fauna as a consequence of endocrine disrupting agents being present at trace levels (Jørgensen, 2000). Since then, surface waters, in particular Sewage Treatment Plants (STP), have been intensively investigated, with the result that a number of relevant drugs vastly used are persistent and can be present at concentrations varying in the range of nanograms to micrograms per liter (Kümmerer, 2001).

There is not yet a clear understanding of the environmental effects and risks involved in the unrestricted use (and discharge) of almost all the drugs presently available in the market. An overview of the present knowledge is given in two review papers, one published in Europe and the other in USA (Halling-Sørensen, 1998; Daughton, 1999). From these papers concern shows through, especially for those substances that can have deleterious effects even at very low concentrations, like hormones and antibiotics, but caution is recommended also for other drugs, for which there is not yet confidence about the absence of unexpected effects in the environment. The

publication of two books and a website specifically dedicated to Pharmaceuticals and Personal Care Products in the environment testifies the increasing interest for this issue (Kümmerer, 2001; Daughton and Jones-Lepp, 2001). According to these sources, the compounds that presently deserve more concern are listed in Table 2.5, while the fate of pharmaceuticals in the environment is presented in Figure 2.4.

For some of these drugs the use as fecal indicators has already been hypothesized. Investigations on caffeine, aspirin, estrogens and certain antibiotics, showed that in wastewater their concentration can reach 300 µg/L, and confirmed the persistence, and therefore the potential of these drugs as general fecal indicators. Indeed, these are just some of the many drugs intensively used in the modern society, and all these substances end up in the sewer and eventually in surface waters.

Because some of these products are used exclusively by humans, and for the most part are excreted with urine and feces, it is not surprising that they could also be very reliable indicators of source fecal contamination. It should be noted that antibiotics are probably the only class of compounds which is used massively both in humans and in animal farming activities. The key of the success of these substances as fecal indicator is in the development of new and specific procedures and methods for the analytical detection. Gas chromatography/tandem mass spectrometry (GC/MS-MS) seems the most promising solution to increase understanding of this complex problem (Sedlak, 2000), and to prove the usefulness of drugs as indicators of

human source of fecal pollution.

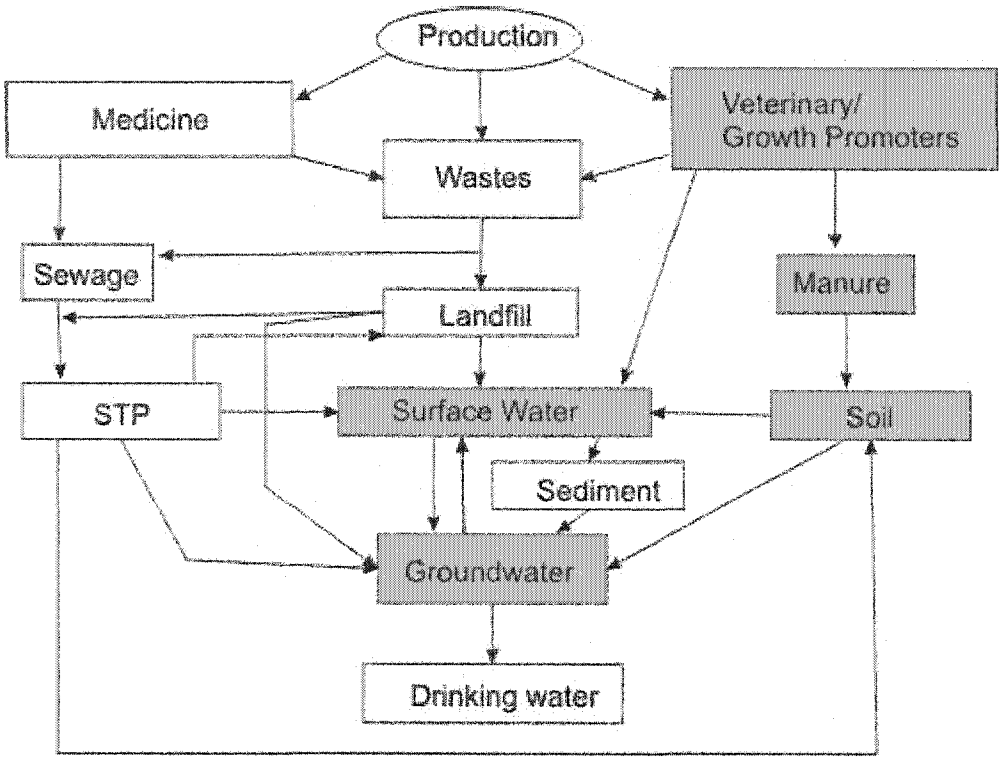


Figure 2.4 – Source, distributions and final destinations of pharmaceuticals in the environment (adapted from Kümmerer, 2001)

In the specific case of groundwater, the low concentrations and the interactions of these drugs with soil are probably the most significant limitations to the use of this source tracking technique.

Table 2.5 – List of PPCP according to USGS (2000).

Veterinary and Human Antibiotics	
Tetracyclines Chlortetracycline Doxycycline Oxytetracycline Tetracycline **Fluoroquinolones** Ciprofloxacin Enrofloxacin Norfloxacin Sarafloxacin **Macrolides** Erythromycin-H2O (metabolite) Tylosin Roxithromycin	**Sulfonamides** Sulfachlorpyridazine Sulfamerazine Sulfamethazine Sulfathiazole Sulfadimethoxine Sulfamethiazole Sulfamethoxazole **Others** Lincomycin Trimethoprim Carbadox Virginiamycin
Human Drugs	
Prescription Metformin (antidiabetic agent) Cimetidine (antacid) Ranitidine (antacid) Enalaprilat (antihypertensive) Digoxin Diltiazem (antihypertensive) Fluoxetine (antidepressant) Paroxetine (antidepressant, antianxiety) Warfarin (anticoagulant) Salbutamol (antiasthmatic) Gemfibrozil (antihyperlipidemic) Dehydronifedipine (antianginal metabolite) Digoxigenin (digoxin metabolite)	**Non-Prescription** Acetaminophen (analgesic) Ibuprofen (anti-inflammatory, analgesic) Codeine (analgesic) Caffeine (stimulant) 1,7-Dimethylxanthine (caffeine metabolite) Cotinine (nicotine metabolite)
Sex and Steroidal Hormones	
Biogenics 17*b*-Estradiol 17*a*-Estradiol Estrone Estriol Testosterone Progesterone *cis*-Androsterone	**Pharmaceuticals** 17*a*-Ethynylestradiol (ovulation inhibitor) Mestranol (ovulation inhibitor) 19-Norethisterone (ovulation inhibitor) Equilenin (hormone replacement therapy) Equilin (hormone replacement therapy) **Sterols** Cholesterol (fecal indicator) 3*b*-Coprostanol (carnivore fecal indicator) Stigmastanol (plant sterol)

d) Antibiotics

As described in the previous sections, antibiotics play an important role both in the microbial and in phenotypic methods of tracking the source of fecal contamination. In the first case, they determine the selectivity of the growth medium in question for a specific microbial species (or group of species), while in the latter the pattern of resistance is used as a "fingerprint" for identification. Besides, they can also be used as chemical tracers, applying the new analytical instruments and techniques available, e.g. GC/MS-MS (Lindsey, 2001). In fact, because the types of antibiotic used in human and animal therapy are often different (the more esteemed and effective antibiotics should be used only for humans), there is the possibility of using them as chemical indicators of a specific source.

This is particularly relevant for those antibiotics used in livestock industry, in particular for those used as growth promoters. There are now many concerns about the effects that the intensive use (or, according to many others, abuse) of antibiotics in farming industry can have in the environment. The major fear is the development of antibiotic resistance in bacteria that are animal pathogens. These microbes could then transfer this resistance (which involves the modification of specific genes) to other organisms infecting humans. This "terror" is testified by the numerous studies dealing with environmental fate and the risk caused by antimicrobial use in the animal farming industry (Mellon, 2001; Veterinary Drugs Directorate, Health Canada, 2002; Jetcar, 1999).

Jørgensen and coworkers (2000)are among the first researchers devoted to this cause. In particular, they developed models to predict the environmental concentrations produced by the use of antibiotics in pig farming. Also they are among the first scientist to use Environmental Risk Assessment (ERA) applied to antibiotics. In a specific study, they predicted (using models) the compartmental concentrations of various growth promoters in soil and water as a consequence of manure spreading activities (Jørgensen, 1998). Certain antibiotics have been found at detectable concentrations in groundwater samples from agricultural areas in Germany (Hirsh, 1999), but there are no studies where antibiotics have been used specifically as MST indicators.

According to the Canadian Ministry of Agriculture and Food (Blackwell, 2003), agriculture is responsible for almost 50 % of all antibiotic use in North America, and 62 % of the antibiotics used in animal production are given to swine (Dunlop, 1998). Considering these estimates, antibiotics could have a prominent role in MST methods. The typical resistance of bacteria populating the intestine of pigs could be used in ARA methods (phenotypic m. see Sec. 2.5.2), while their presence can be used as chemical tracers and directly detected in waters. Nonetheless, these alternatives have not been investigated yet.

Chapter 3

Experimental Procedures

The experimental procedures adopted for this study are described in this chapter. First, the enumeration methods are described in detail, and then the experimental phases are presented by illustrating all the activities accomplished.

3.1 – Description of the Enumeration Methods

To distinguish human from animal sources of fecal contamination three different methods based on the enumeration of bacterial indicators have been used:

1. **Fecal Coliforms to Fecal Streptococci ratio**: according to some authors (Geldreich, 1969) when this ratio is greater than 4, it indicates a human source, while when it is less than 0.7 the source is animal.
2. ***Clostridium perfringens***: Although there are still many controversies, according to some authors this species can be used to distinguish animal manure from septic system pollution sources in groundwater (Conboy, 2001).
3. **Sorbitol Fermenting Bifidobacteria**: according to some authors (Mara, 1983), the presence of this group of bacteria should be ascribed only to a human source of contamination.

Together with these bacterial indicators, **Total Coliforms** have also been enumerated as a useful reference to control for the general level of fecal pollution in the samples analyzed. The enumeration of the entire bacterial indicators has been done by using membrane filtration.

The Membrane Filtration Test

The Membrane Filtration test is a technique commonly used to determine the number of bacteria belonging to a species (or to a specific family or group of species) in a water sample (Maier, 2000). In this test, a membrane filter having a pore size of 0.45 μm is placed on a filter unit, and a measured volume of water is passed through the filter. The bacteria get trapped onto the membrane. After the sample has been filtered, the membrane is removed from the filter holder and it is placed on a Petri dish filled with a specific medium. Incubation at a specific temperature and for a specified time allows the growth of a single species (or group) up to a size visually detectable (and therefore countable). The colonies having the expected color and morphology for the given medium are counted, and the concentration is then reported as colonies forming units per 100 milliliters (CFU/100mL) or per liter (CFU/L).

The procedure for the Membrane Filtration test can be divided in two parts. In the first part, the following materials are needed:

- Forceps
- Gas burner
- Pipettes of the desired volume and pipette bulb

- Flask with 1 liter of sterilized water
- Vacuum source and vacuum hose
- Filter unit, which is composed of a flask attachable to a vacuum source, a filter funnel and a sterile top
- Sterile 0.45 µm pore, 47 mm diameter filters
- Sterile 50 x 12 mm Petri dishes prepared with the desired growth medium.

The procedure of filtration, which should be applied independently of the bacterial indicator, is the following (Pepper, et al., 1995):

1. Prepare the Petri plates with the desired growth medium adopting aseptic procedures (if the medium is agar this operation can be done several days in advance).
2. Assemble the filtration unit taking precautions to avoid contamination of the funnel top.
3. Remove the funnel top and place a sterile membrane filter centering it in the filter-holder using flamed forceps[ii], then place the funnel top.
4. Pour about 50 mL of sterile water (buffer-water) on top of the filter, and then pipette the desired volume of sample into the funnel.
5. Apply the vacuum gently, and as the liquid reaches the filter,

[ii] The forceps should be sterilized after each handling using ethyl alcohol for flame sterilization or the flame source directly, but in this case it is better to wait until the forceps are cold enough in order to avoid damage to the membrane.

rinse the sides of the funnel top with a small amount of sterile water, then let the vacuum draw all the water through the filter. Avoid squeezing water directly on the filter.

6. Remove the funnel top with the vacuum still applied and remove the membrane filter carefully with sterile forceps.
7. Apply the filter face-up directly on the medium prepared in the Petri plates, and then label the plate.
8. Repeat steps 2 to 7 using a different growth medium, or filtering a different volume of water sample (or both).
9. Incubate the dishes inverted (bottom-up) at the temperature and for the time specified for the BI.

In the case of enumeration of anaerobic species, the plates should be placed inverted into an anaerobic jar together with an anaerobic gas generating envelope. The jar is then sealed and placed in the incubator. After half an hour, check whether the pressure of the jar has increased. In case it has not, the jar should be opened and the anaerobic gas generating envelope should be replaced with a new one.

In the second part the following materials are needed:

- Incubated plates from the first period
- Dissection microscope or magnifying lens
- Counter-pen,

and the following procedure should be followed:

1. Examine the plates under the microscope and identify the colonies that have the color and morphology identical to those described in literature for the specific growth medium.
2. Count the colonies that have been identified as corresponding to the description marking them with the counter-pen on the lid of the plate.

Depending on the Bacterial Indicator, it is necessary to proceed with the confirmation test for the specific organism. This procedure will be discussed later.

Growth media, incubation conditions and confirmation tests

The basic idea of the membrane filtration test is to culture the target bacteria using specific nutrients and specific growth conditions. The following parameters are fundamentally important in this technique:

- Nutrients present in the medium: their ratio should allow an optimal growth of the target species
- Environmental conditions: such as presence or absence of oxygen and humidity. Some bacteria are aerobic, some facultative, while some others are strictly anaerobic. Accordingly, the latter should be incubated in an anaerobic jar or similar device. The optimal humidity of the incubator should be around 50-60 %. If the air inside the incubator is too dry it will affect the growth medium in the plates, reducing the efficiency of growth medium.

- Temperature and time of incubation: these should be chosen in order to obtain an optimum growth of the colonies up to a visible size. An excessive incubation time should be avoided in order to diminish the probability of fusion of different individual colonies that may become indistinguishable (the plate in this case is defined as "over-grown").

Often, more than one species or groups of bacteria present in the sample are characterized by the same optimal growth conditions (both the type of nutrients and the incubation conditions) or these are very close. In this situation, another important factor that could affect the selectivity of the medium for the target species is the addition of antibiotics and other growth inhibitors specific for the unwanted species. In fact, whenever the target BI has a higher resistance to one or more antibiotics[iii], compared to the other interfering species, it is convenient to add to the medium this substance at a concentration which is higher than the concentration inhibitory to the interfering species, but lower than that inhibitory to the target bacteria. Technically, the value of concentration of antibiotics in the medium should be between the Minimal Inhibitory Concentrations (MIC) of the two species, where the higher MIC should be that of the indicator.

As mentioned previously, five different bacterial indicators have been selected for the enumerations performed in this research. The media and the incubation conditions specifically used in this study are described below (see

[iii] It is frequent the use of cocktails of 2 or 3 antibiotics.

Appendix A1 for details on the preparation procedure):

Total Coliforms

Medium: M-Endo with 2 % of ethanol 95 % [Millipore Ltd]

Incubation conditions: 35 °C ± 0.5 °C for 22 to 24 h

Identification method: the colonies are red or pink showing a golden-red metallic sheen (see Figure 3.1 – b)

Fecal Coliforms

Medium: M-FC with 1 % rosolic acid in 0.2 N NaOH [Millipore Ltd]

Identification method: 44.5 °C ± 0.2 °C for 22 to 26 h

Counting procedures: the colonies are blue (see Figure 3.1 – c)

Fecal Streptococci

Medium: KF streptococcus agar with 1 % triphenyltetrazolium chloride (TTC) [Millipore Ltd]

Identification method: 35 °C for 48 h

Counting procedures: the colonies are small and bright red (see Figure 3.2 – a)

Clostridium perfringens (*medium 1*)

Medium: SFP agar with addition of antibiotics kanamyxin sulfate and polymyxin B sulfate (BD)

Incubation conditions: 37 °C for 48 h in anaerobic jar

Identification method: according to the specification given by

the producer of the medium, the *Clostridium perfringens* colonies are black with halo (see Figure 3.2 – b; see Appendix A1 for further description).

The SFP agar is usually used for detecting *C. perfringens* in food. The only documented attempt of using this medium for the analysis of water samples is that of Conboy and Goss (2001).

In this study, the use of SFP agar was attempted in conjunction with the membrane filtration test. Encouraging results were obtained using dilutions of *C. perfringens* pure species. Black colonies were obtained when there was a sufficiently high dilution of the sample so that no more than about 80 colonies fixed to the membrane. With an increasing number of bacteria fixed on the membrane, the colonies grew smaller in size and beige in color. This effect can be visualized in Figure 3.1 - a.

Less encouraging results were obtained by filtering dilutions of manure. In this case, there were significant interferences with other species of bacteria, which were able to grow because of the low selectivity of this medium. For these reasons, the SFP agar combined with the MF test was used only for the enumeration of the pure species in Phase III. In other cases (Phases II and IV) mCP, which is described in the following paragraph, was adopted. The effectiveness of these two growth media is described later.

Clostridium perfringens (*medium 2*)

Medium: mCP modified, as described by Armon and Payment (1988) [prepared in the lab using pure chemicals from accredited suppliers]

Incubation conditions: 45 °C for 18 to 24 h in anaerobic jar

Identification method: according to Bisson and Cabelli (1979) (see also EPA/600/R-95/178, US-EPA, 1996) for the enumeration of *C. perfringens,* colonies the following procedure was followed:

- Examined the mCP plate for straw-yellow colonies
- If such colonies were present, invert and expose the open agar plate for 10-30 seconds to the fumes from an open container of concentrated ammonium hydroxide (28 % NH_4OH)
- If *C. perfringens* colonies were present, the typical colonies turned a dark pink of magenta after exposure to the fumes
- Counted pink of magenta colonies as presumptive *C. perfringens* (see Figure 3.2 – d; see Appendix A1 for further description).

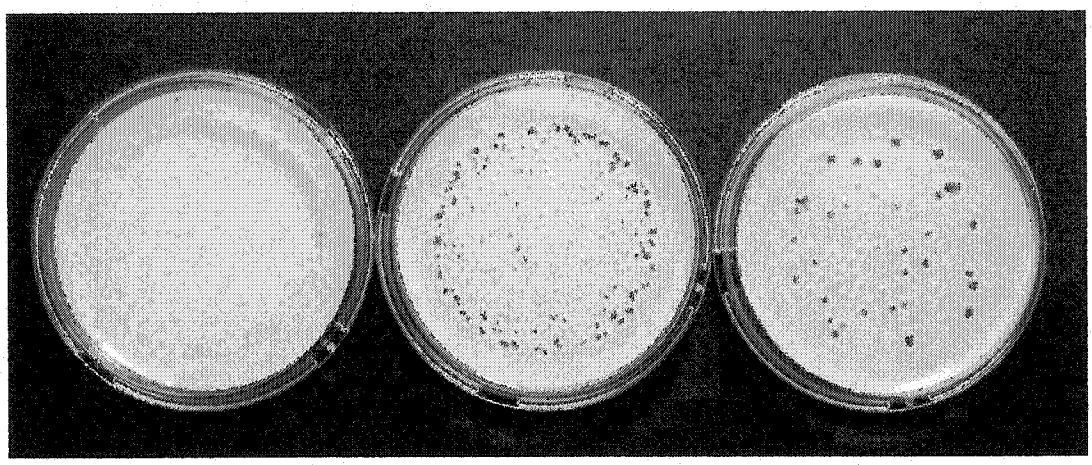
The effectiveness of these two growth media were evaluated using dilutions of sterile buffered water containing *C. perfringens* pure species. In this study it was observed that the recovery of this species in SFP was about 20 % higher than in mCP.

Sorbitol Fermenting Bifidobacteria

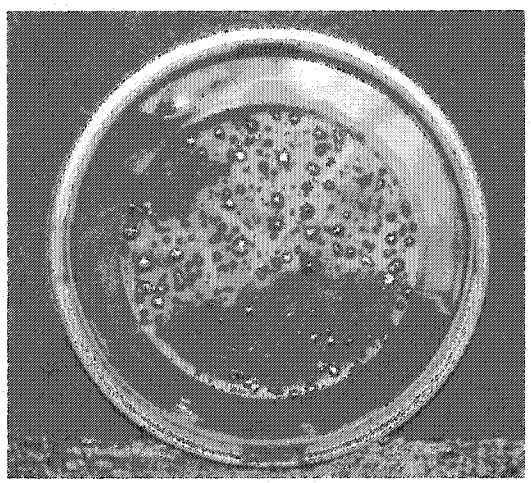
Medium: Human Bifidobacteria Sorbitol Agar (HBSA), as described by Mara and Oragui (1983) [prepared in the lab using pure chemicals from accredited suppliers]

Incubation conditions: 37 °C for 48 h in anaerobic jar

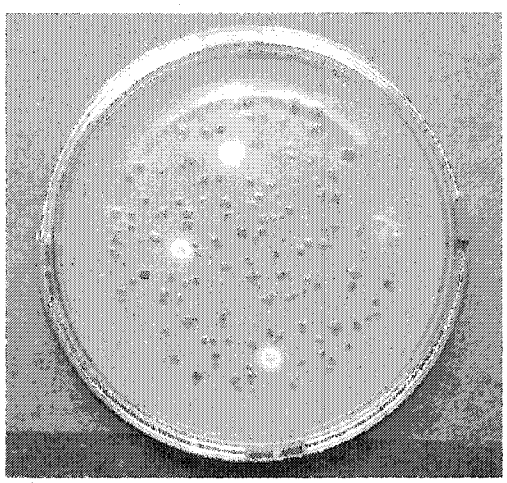
Identification method: according to the references (Mara, 1983) the colonies of *Bifidobacterium adolescentis* and *Bifidobacterium breve* are yellow (see Figure 3.1 – c; see Appendix A1 for further description).



a) Decreasing numbers of *C. perfringens* fixed to the membrane (decreasing volume of sample filtrated, 100/10/1). It can be easily noted that a high number of CFU did not allow the development of black colonies (the colonies are dark-yellow to beige).

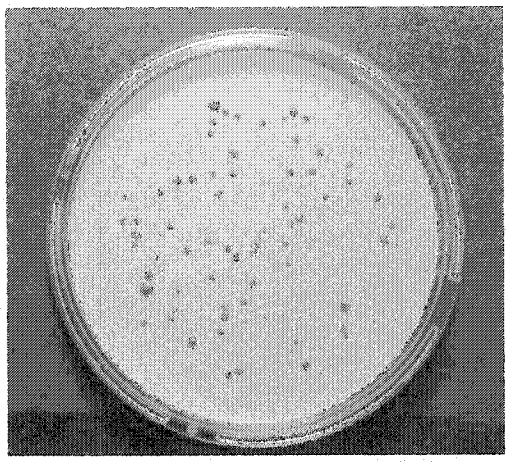


b) Total Coliforms

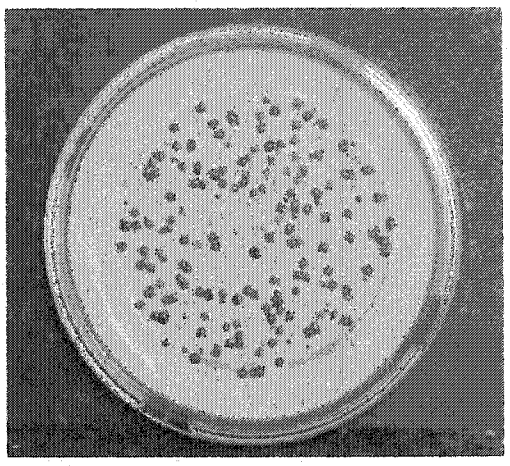


c) Fecal Coliforms

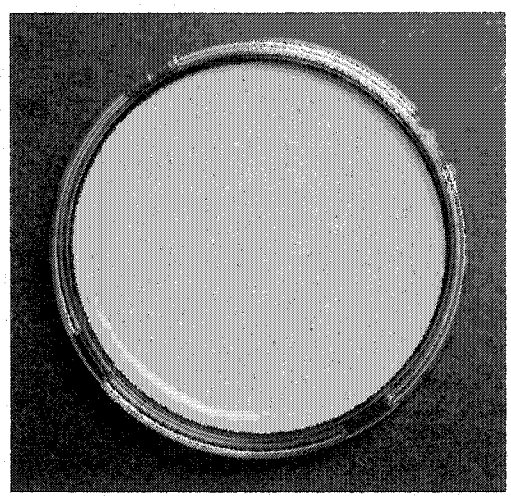
Figure 3.1 – Appearance of BI



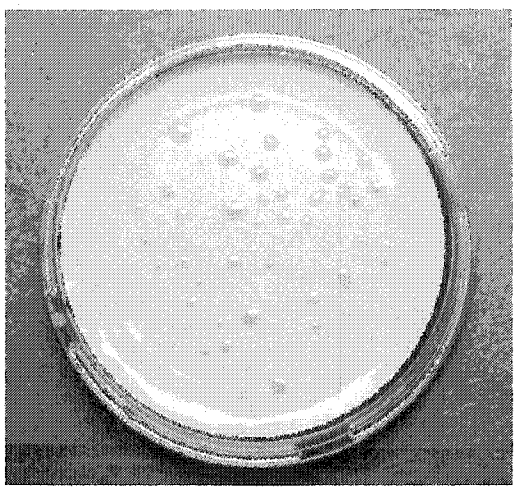
a) Fecal Streptococci



b) *C. perfringens* in SFP



c) Sorbitol F. Bifidobacteria



d) *C. perfringens* in mCP

Figure 3.2 – Appearance of BI

After the enumeration of Clostridium perfringens and Sorbitol Fermenting Bifidobacteria, a selected number of samples were chosen for confirmation tests. The confirmation tests for the specific bacterial indicators are described below:

Clostridium perfringens

The confirmation tests proposed by EPA (EPA/600/R-95/178, US-EPA, 1996) are Gram stain test and Iron Milk medium test, and the following procedures were performed:

a. Picked at least 10 presumptive colonies of *C. perfringens* from the plate and transferred each isolate into a separate tube filled with thioglycollate (a non selective clostridial medium).
b. Incubated at 35 °C for at least 24 h.
c. Examined by Gram strain and for purity.
d. Inoculated ten tubes of iron milk medium with 1 mL from the ten tubes prepare in a) above and incubated at 44.5 °C for 2 h.
e. Examined hourly for stormy fermentation of milk with rapid coagulation and fractured rising curd.
f. Those colonies which were Gram-positive, non-motile and produced stormy fermentation of milk in these confirmatory tests were considered as confirmed *C. perfringens.*

Sorbitol Fermenting Bifidobacteria

The confirmation tests proposed by Mara and Oragui (1983) are Gram stain test and glucose or lactose fermentation tests. Presumptive

Bifidobacteria showed typical Gram-negative bulbate bifurcated Y or V forms. They were catalase negative, non-motile and fermented glucose or lactose without gas production.

For the identification of the anaerobic species, another method was used. The BBL Crystal™ Identification System – Anaerobe ID Kit, which is a miniaturized identification method employing modified conventional, fluorogenic and chromogenic substances, intended for the identification of frequently isolated anaerobic bacteria. The BBL Crystal anaerobe ID System also required a Gram stain, catalase and indole test results (See Appendix A2 for a detailed description of these tests).

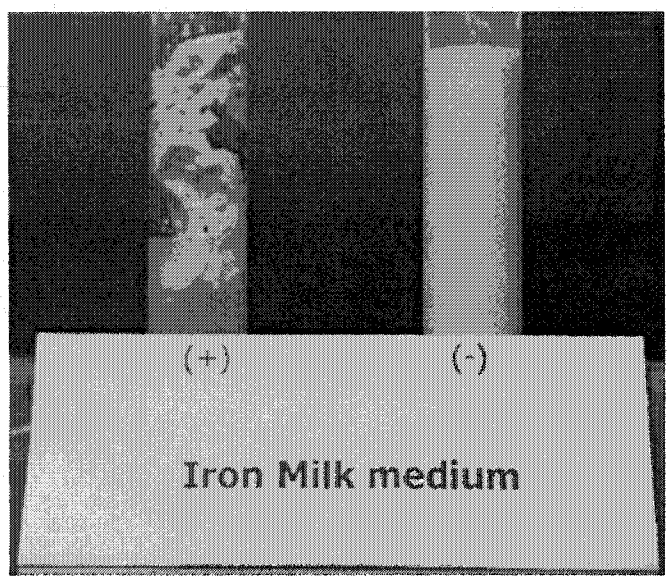


Figure 3.3 – The iron milk medium test

3.2 – Description of the Experimental Phases

As stated in the introduction, the objective of this study was to evaluate and compare the effectiveness of three selected methods of Microbial Source Tracking (MST) in distinguishing human from animal fecal contamination in groundwater (the methods are Fecal Coliforms/Fecal Streptococci ratio; *C. perfringens*; Sorbitol fermenting Bifidobacteria), and then to try to apply the methods in real ground water samples.

In order to accomplish these tasks, the experimental activities have been organized in the following phases:

Phase I. Testing of the ability to distinguish human from animal microbial contamination for the three methods selected in real samples.

Phase II. Observation over a period of 60 days of the behavior of the Bacterial Indicators in three simulated groundwater micro-environments, using fresh inoculum of pig manure, stored manure from a cesspit of a pig farm, and wastewater taken from a septic system.

Phase III. Determination of the die-off coefficients for *Clostridium perfringens* and *Bifidobacterium adolescentis* pure species in a groundwater micro-environment.

Phase IV Application of the selected MST methods in samples of water taken from wells located in rural areas.

3.2.1 – Phase I. Testing the three methods

In this phase, the set of Bacterial Indicators was tested to evaluate the capacity to distinguish between human and animal microbial contamination. The samples tested were:

- 1 sample of fresh pig manure diluted in sterile buffer solution
- 1 sample of manure stored in a cesspit of a pig farm
- 1 sample of wastewater taken from a septic system
- 2 samples of effluent of Wastewater Treatment Plant

The samples were provided by Mr. David Armstrong (manure samples) and Mr. Peter Bitzer of the L. Romano Wastewater Treatment Plant in Windsor (septic system and WWTP effluent samples).

The media for *C. perfringens* and Sorbitol Fermenting Bifidobacteria were tested previously on the pure species. Each test was performed at least twice per sample, the first are to find the range and the second are to measure the actual concentration.

The objective of this phase was to identify the most effective MST method among the three selected.

3.2.2 – Phase II. Observation of the behavior of BI

In this phase, three groundwater simulated micro-environments (GSME) were set up. Each GSME was composed of a Pyrex flask of 2 gallons (9.1 L) filled with sterilized groundwater and inoculated with a given quantity of sample containing bacteria. The flasks were wrapped with aluminum paper

to maintain a dark environment and stored in a refrigerator at a constant temperature of 8 °C.

The procedure of preparation of each flask involved the following steps: an initial wash of the flask with a solution of nitric acid, followed by abundantly rinsing with tap water; a second wash with detergent and a rinse with tap water; finally a rinse with deionized water.

After the preparation, each dried flask was filled with ground water and autoclaved at 121 °C for 15 minutes. When the flask was cold, the air was stripped by a flux of nitrogen (N_2) bubbling into the flask through sterilized plastic tip until the concentration of dissolved oxygen reached the wanted value. The dissolved oxygen was measured using YSI 85 portable DO-meter. Then, the flask was immediately sealed with a rubber stopper sterilized in ethyl alcohol. Finally, the flask was wrapped with aluminum paper, labeled, and stored in the refrigerator until the final temperature was reached. After this preparation, the flasks were ready to be inoculated.

Each flask was finally inoculated with one of these samples:

- diluted of fresh pig manure (Experiment **P1**)
- manure stored in a cesspit of a pig farm (Experiment **P2**)
- wastewater taken from a septic system (Experiment **S**)

The volumes of inoculation used are specified in Table 3.1. The dilution of fresh pig manure (Exp. **P1**) was prepared by diluting 8 grams of feces in 2 L of sterile phosphate buffered dilution water in a 4 L Erlenmeyer flask. This

suspension was mixed for 30 minutes by using a magnetic stirrer, and then it was aseptically filtered to remove suspended solids by using Filter Paper P8 qualitative. The filtrate was then used to inoculate the flask labeled **P1**.

The other two liquids of inoculation (Exp. **P2** and Exp. **S**) were not pre-treated but were directly added to the flasks. The characteristics of the water inside each flask at the beginning of the experiment are reported in Table 3.2.

Table 3.1 – Volumes of wastewater inoculated

Flask	**P1**	**P2**	**S**
Volume [mL]	200	500	200

Table 3.2 – Characteristics of the water in the flasks

Flask	**P1**	**P2**	**S**
Temperature [°C]	8	8	8
Dissolved oxygen [mg/L]	< 0.8	< 0.8	< 0.8
pH	7.00	6.99	7.00
Suspended Solids [mg/L]	65	156	80
Turbidity [NTU]	33.4	121	32.9

The reason for the higher turbidity value in the flask **P2** was due to the characteristics of the corresponding waste used to inoculate; namely liquid manure from cesspit.

The initial characteristics of the groundwater used in the three experiments are reported in Appendix A3.

After being inoculated, the flasks was kept in the refrigerator at a constant temperature of 8 °C for a period of 70 days during which samples were taken for analysis.

The sampling procedure implied simply the removal of the flask from the refrigerator, and the withdrawal of the samples using sterilized pipettes. All the operations were performed so as to minimize variations in temperature and to avoid increase in the dissolved oxygen concentration[iv]. The samplings were performed weekly over a period of 60 days. For each sample, the following bacterial indicators were enumerated:

- Total Coliforms
- Fecal Coliforms
- Fecal Streptococci
- *Clostridium perfringens* (using mCP medium)
- Sorbitol fermenting *Bifidobacteria*.

The objective of this phase was to observe the effectiveness in distinguishing sources of fecal contamination for the MST methods selected

[iv] Dissolved Oxygen concentration was measured after 1 month and at the end of the experiment. The observed variations were insignificant.

under conditions similar to those typical for groundwater in the area where the water sample was taken (Southwestern Ontario). See Sec. 4.2 for the results.

This experimental set-up had the following limitations:

1. The characteristics of the water used can be considered representative only of the shallow aquifer in which the well is located (see Appendix A7 for description of location).
2. Using this experimental design, the effects of soil, or solid/liquid interfaces, were neglected. Nevertheless, the porous media can have a very important role in the fate and persistence of microorganisms in groundwater.
3. The effects of the microflora of the groundwater environment were neglected. Indeed, other bacteria or microorganisms might have a decisive influence on the BI (e.g. predation by protozoa, etc...).
4. The effect of nutrients was not studied.

3.2.3 – Phase III. Determination of the Die-off coefficients

In this phase, six groundwater simulated micro-environments were set up. Each of these GSME consisted of a Pyrex flask of 4.55 L (1 gallon) prepared by using the same procedure as described in Phase II. Two flasks were inoculated with a dilution of *Clostridium perfringens*, two with *Bifidobacterium adolescentis*, and the remaining two with Sorbitol Fermenting

Bifidobacteria[v]. The experiments were run in duplicate: 2 for *C. perfringens*, 2 for *B. adolescentis* and 2 for SFB.

The *C. perfringens* culture was supplied by the Department of Biological Sciences of the University of Windsor, while the culture of *B. adolescentis* was supplied by the American Type Culture Collection (ATCC # 15703) (see Appendix A4 for detail on the reviving of the culture). The culture of Sorbitol Fermenting Bifidobacteria was obtained from the septic system wastewater (sample D in Phase I), culturing a yellow isolate grown on an HSBA plate. The colony was picked up using a sterile cotton swab and transferred to a culture tube. In all cases, the species were grown in sealed culture tubes using reinforced clostridial medium (BBL) at 37 °C.

To inoculate the flasks, concentrated solutions of bacteria were prepared by filtering the content of the tubes in sterile conditions (Filter Paper P8 qualitative; Fisher Scientif.). The filtrated was centrifuged in sterilized tubes at 500 *g* for 10 minutes (Husman, 1993). The supernatant was discarded and the pellets were resuspended in 500 mL of sterile phosphate buffered dilution water. The concentration of bacteria was estimated for each dilution, and finally a volume sufficient to obtain a concentration of 10^{+6} ~ 10^{+7} CFU/L was added to the flasks.

The samples were collected every week and for a period up to 50 days to determine a death rate constant. The same procedures described in Phase

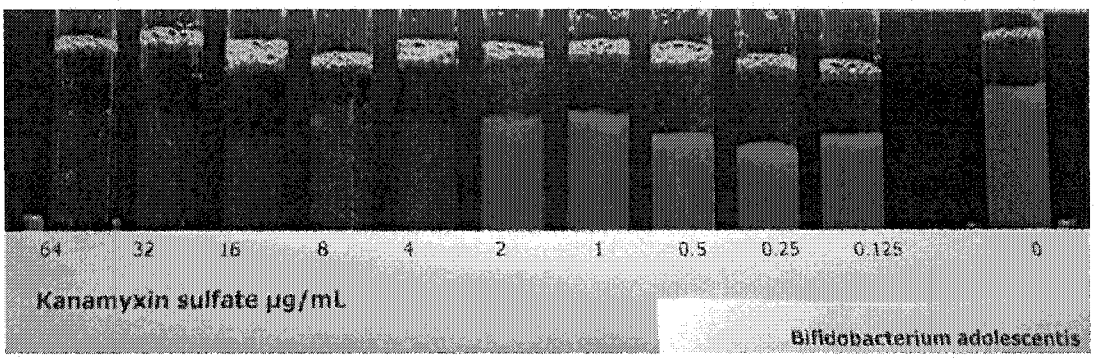
[v] This experiment was performed because *B. adolescentis* supplied by ATCC showed sensitivity to one of the antibiotics contained in HBSA; therefore, it was assumed that the SFB isolate would have been more representative of a real fecal contamination event.

II were used for each sample taken. The corresponding bacterial indicator was measured in triplicate.

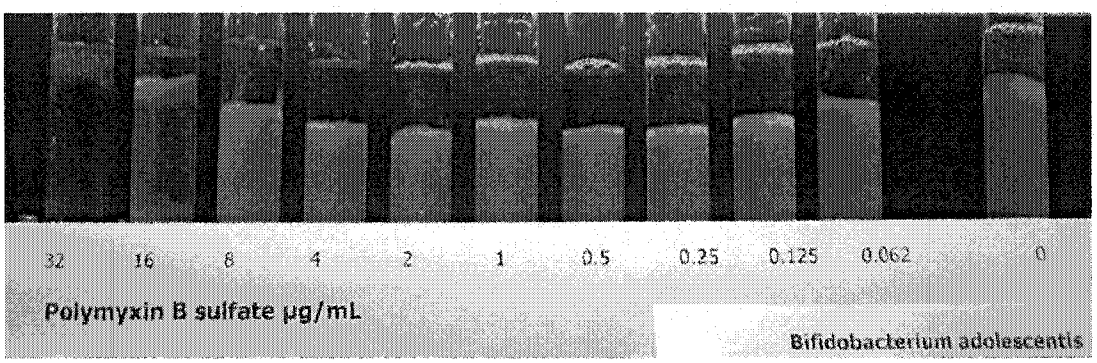
The medium used to enumerate *C. perfringens* was SFP agar (see Appendix A1) and the black and beige colonies were counted. The medium used to enumerate *B. adolescentis* was HBSA without antibiotics and the yellow colonies were counted. The medium used to enumerate SFB was HBSA agar (complete), prepared as indicated by Mara (1983).

For the enumeration of *B. adolescentis* pure species, the medium (HBSA) was prepared without the antibiotics kanamyxin sulfate and polymyxin B sulfate because during this experiment it has been noted that this species did not grow in the regular HBSA medium. After a successful attempt to culture this bacterial species in a tube filled with HBSA broth without antibiotics, the Minimal Inhibitory Concentrations (MIC) of these two antibiotics for *B. adolescentis* was measured to verify whether the concentration used in HBSA was too high (see Appendix A3; see Figure 3.4). A similar result was obtained by Rhodes (1999).

The results obtained showed that the concentration of kanamyxin sulfate in HBSA medium was 25 times the MIC for the strain used in these experiments (ATCC # 15703). In particular, the MIC for *B. adolescentis* was 2-4 µg/mL for kanamyxin sulfate, and 16 µg/mL for polymyxin B sulfate. The concentrations suggested by Mara (1983) for HBSA are 50 µg/mL for kanamyxin sulfate and 1.23 µg/mL for polymyxin B sulfate.



a)



b)

Figure 3.4 – Determination of MIC for B. adolescentis.

The objective of this phase was to measure the persistence of the two species in conditions similar to typical groundwater in the area where the water sample was taken (Southwestern Ontario). This information is fundamental to assess the effectiveness of a BI as indicator of fecal contamination (see point 5 in Sec. 2.5). Furthermore, the die-off coefficients of these two species for the typical groundwater conditions were not available in literature. The limitations of this phase were similar to those describe previously for Phase III.

3.2.4 – Phase IV. Application with groundwater

In this phase, the complete set of Bacterial Indicators was measured in the two samples of water taken from a well located in Ridgetown (provided by Dr J. Laird), and in a sample of water taken from a well located in Essex (provided by Mr. E. Manzocco). These samples were collected in August and November 2003 according to the procedures defined in Standard Methods (1998), while the enumerations according to the procedure described in Phase I.

The objective of this phase was to try the MST methods in field samples, and to observe differences in performance of the methods with respect to the GWSM experiments of Phase II.

Chapter 4

Results and Discussion

The results obtained in this study are presented and discussed in this chapter. The order of presentation corresponds to the sequence of the experimental phases, namely:

I. Testing of the three MST methods in real manures and wastewater samples.

II. Observation over a period of 60 days on the behavior of the BI in the three simulated groundwater micro-environments (flasks labeled P1, P2 and S).

III. Determination of the death rates for *Clostridium perfringens* (flasks labeled Cp α and Cp β), *Bifidobacterium adolescentis* (flasks labeled Ba γ and Ba δ) and Sorbitol-fermenting Bifidobacteria (flasks labeled SFB α and SFB β) in a groundwater micro-environment.

IV. Application of the MTS methods in samples of water taken from wells located in rural area.

4.1 – Phase I

In this phase, three selected methods, namely FC to FS ratio, *C. perfringens* (CP), and Sorbitol fermenting *Bifidobacteria* (SFB), were tested on the following samples:

- 1 sample of fresh pig manure diluted in sterile buffer solution (sample A)
- 1 sample of manure stored in a cesspit of a pig farm (sample B).
- 1 sample of wastewater taken from a septic system (sample C)
- 2 samples of effluent of Wastewater Treatment Plant (samples D and E).

Total Coliforms (TC) were enumerated together with the other bacterial indicators. The results are reported in Table 4.1.

Table 4.1 – Results of Phase I

Bact.Ind.	**TC**	**FC**	**FS**	**FC/FS**	**CP**	**SFB**
Sample	CFU/100mL	CFU/100mL	CFU/100mL		CFU/100mL	CFU/100mL
A[vi]	9.7E+05	2.7E+05	2.7E+05	1.00	1.0E+05	< 1
B	1.6E+05	1.9E+04	9.6E+04	0.20	4.0E+04	< 1
C	1.5E+06	4.3E+04	4.1E+06	0.01	9.0E+04	1.1E+05
D	2.5E+06	3.6E+04	2.7E+04	1.34	1.0E+04	2.7E+05
E	4.5E+06	3.2E+06	3.5E+05	9.14	2.0E+04	6.0E+04

[vi] The concentration of BI as #CFU/g is equivalent to 0.4 #CFU/100mL.

The concentrations of the different BI obtained for each sample and the ratios of their values can be visualized in Figure 4.1 and compared to the estimated average values found in Raw Sewage (RSW) (adapted from Gerba, Chap. 20 in Maier, 2000).

It should be pointed out that **CP** was present in all the samples, both of human and animal origin, at concentrations ranging from 10^{+4} to 10^{+5} CFU/100 mL. Therefore, it cannot be considered a good indicator of the source of fecal contamination.

It is noted that only in sample B (manure from cesspit) and E (effluent form WWTP) **FC to FS ratio** is accurate in the determination of the source of fecal pollution. On the contrary, in sample A (fresh pig manure), and C and D (septic system and WWTP effluent) the results do not indicated the actual condition.

In this Phase, the use of **SFB** as an indicator of human source of fecal contamination resulted in this case the most reliable. In fact, this group of bacteria was found only in the samples containing human feces (C, D and E). Although this test was accurate in the identification of the source, it was observed that other colonies were able to grow on the HBSA plates. Thus, the medium was not enough selective since competition among bacterial species was also noted (the different colonies grew in clusters). It is very likely that these factors interfered with the regular growth of SFB, and, therefore, the concentrations obtained were not representative.

It is also observed that the ratios of TC, FC and FS for sample A are not in accordance with the average densities reported for pig feces (Gerba, Chapter 20 in Maier, 2000). In this case, the number of FC was higher than expected. The probable reason for this result may be in the diet or in the different farming conditions for the pigs that produced the feces samples used in this study.

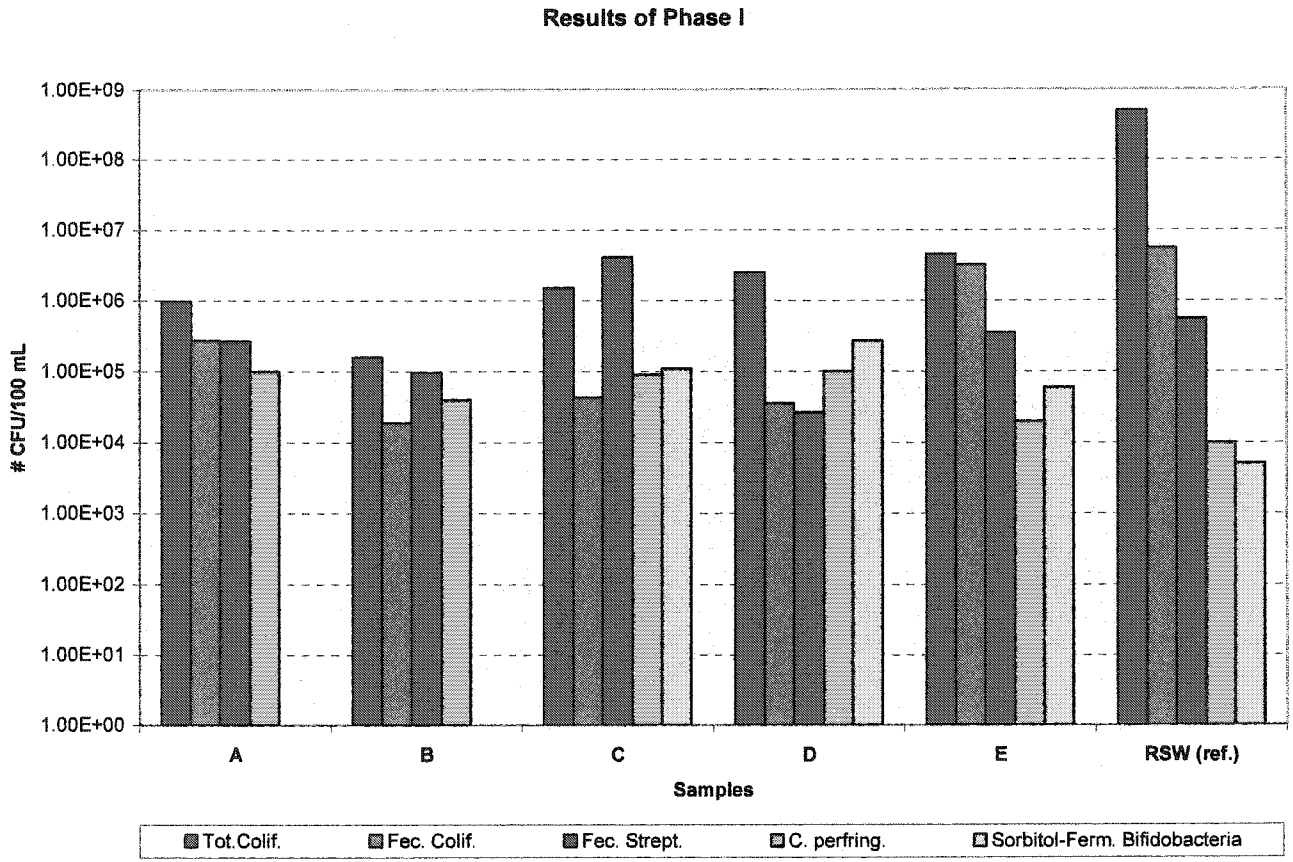


Figure 4.1 – BI in phase I, where A = dilution of fresh pig manure; B = manure from cesspit; C = wastewater from septic system; D, E = effluent from WWTP; RSW = estimated level of BI in Raw Sewage (Maier, 2000)

4.2 – Phase II

In this phase, three flasks, containing sterilized groundwater taken from a well located in Ridgetown (see Appendix A7), were inoculated with:

- Diluted fresh pig manure (labeled Experiment **P1**)
- Manure stored in a cesspit of a pig farm (labeled Experiment **P2**)
- Wastewater taken from a septic system (labeled Experiment **S**).

Samples of water were withdrawn every week from each flask over a period of more than 60 days and the BI were enumerated. The results obtained for Experiment **P1** are reported in Table 4.2 and in Figure 4.2.

Table 4.2 – Concentrations of BI for microenvironment P1

Bact.Ind.	**TC**	**FC**	**FS**	**FC/FS**	**CP**	**SFB**
Days	CFU/100mL	CFU/100mL	CFU/100mL		CFU/100mL	CFU/100mL
0	9.5E+03	2.4E+03	1.0E+03	2.4	6.0E+02	< 1
10	8.0E+03	5.0E+04	8.0E+02	0.6	1.0E+02	< 1
17	7.2E+03	2.0E+03	1.1E+03	1.9	n.d.	< 1
24	6.5E+03	1.1E+03	3.0E+02	3.8	2.0E+02	< 1
31	3.7E+03	2.0E+03	7.6E+02	2.6	8.0E+01	< 1
38	3.0E+03	1.9E+03	6.3E+02	3.0	2.0E+01	< 1
45	2.6E+03	1.2E+03	9.0E+01	13. 9	6.0E+01	< 1
52	1.10E+03	1.0E+03	4.0E+02	2.5	3.4E+01	< 1
59	1.1E+03	9. 7E+02	2.3E+02	4.2	3.4E+01	< 1
66	1.3E+03	1.2E+03	1.7E+02	7.6	2.8E+01	< 1

n.d. = not determined

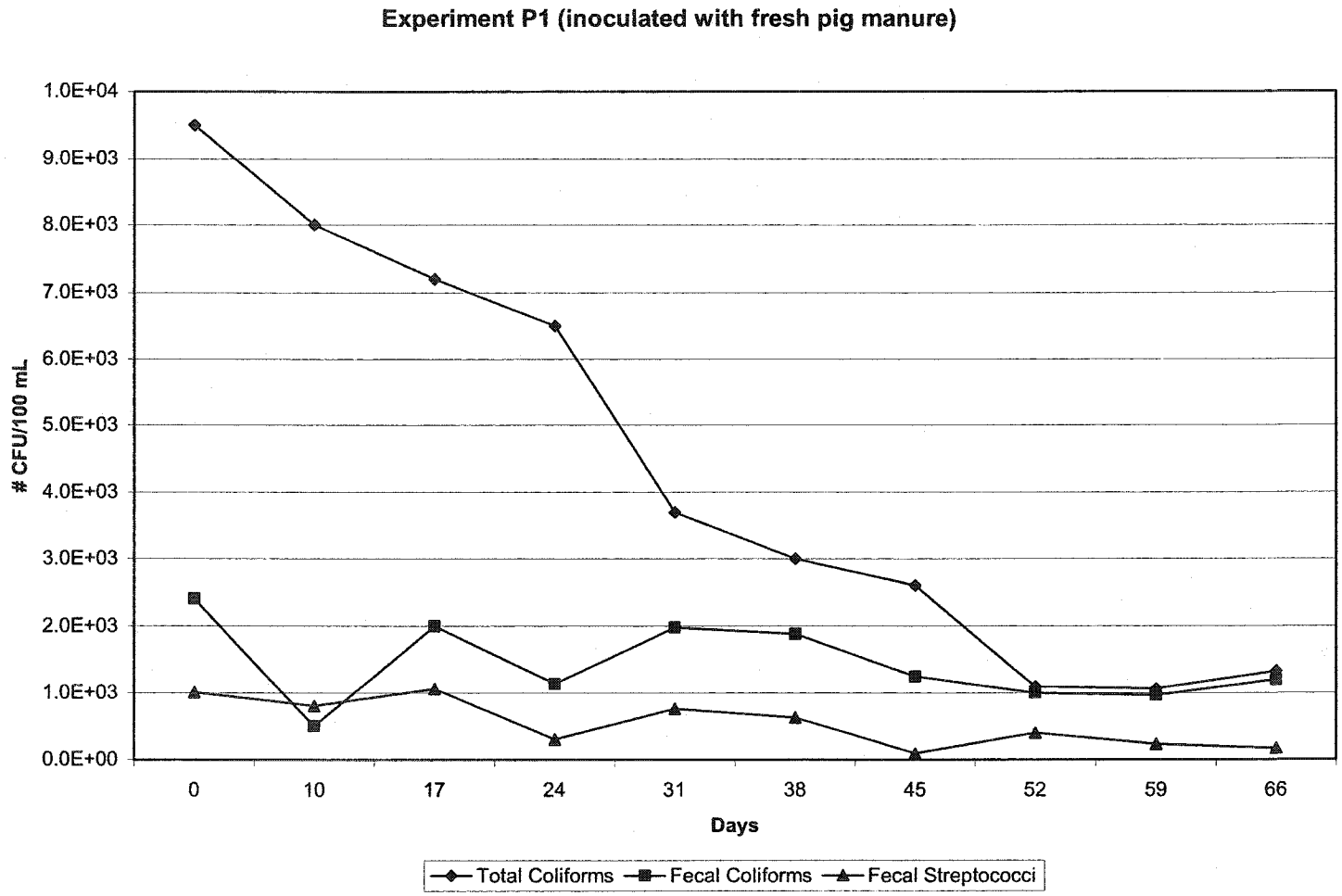


Figure 4.2-a – Decay of BI in Experiment P1 with time.

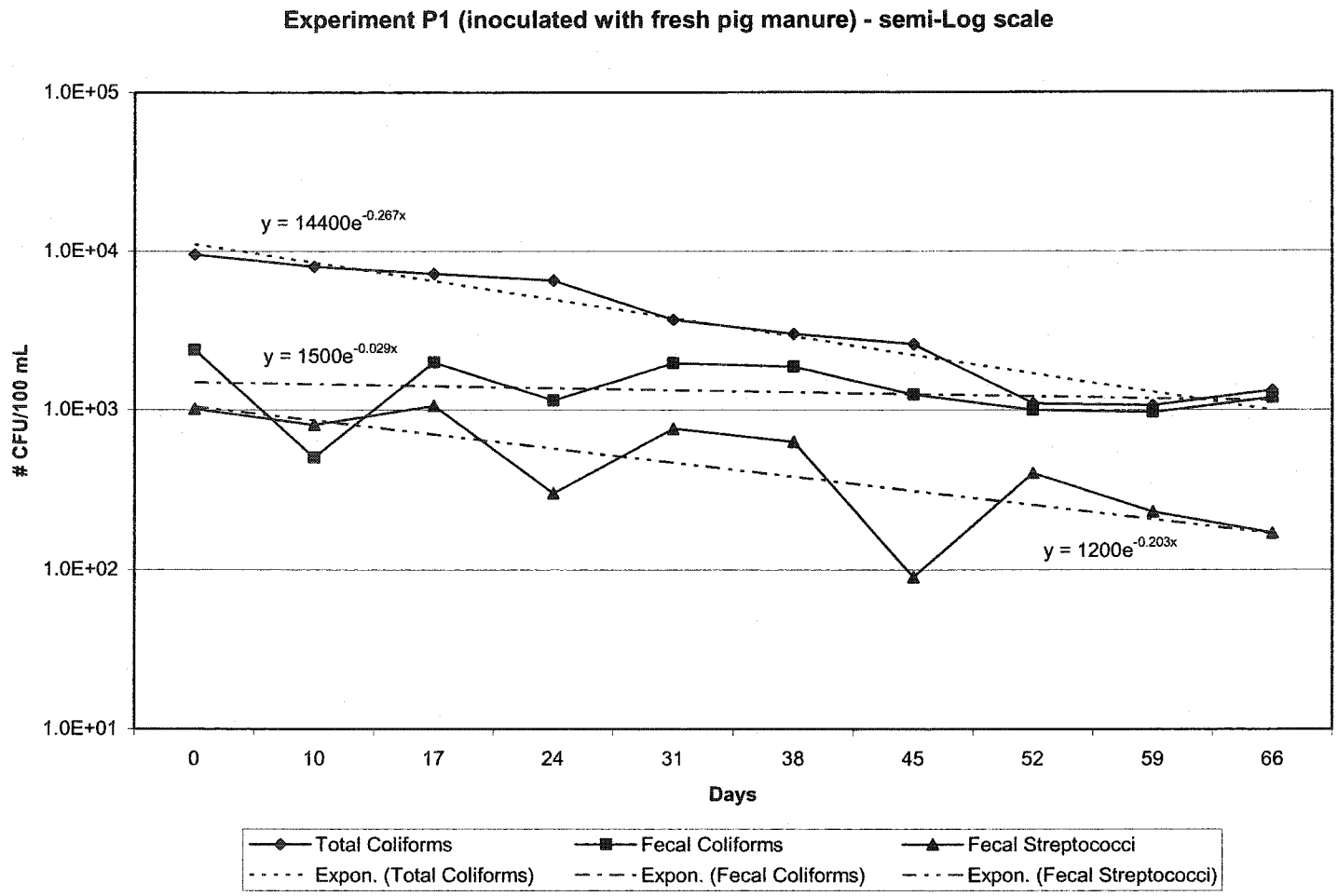


Figure 4.2-b – Decay of BI in Experiment P1 with time and exponential fitting in semi-Log scale.

The results obtained for Experiment P2 are reported in Table 4.3 and represented in Figure 4.3.

Table 4.3 – Concentrations of BI for microenvironment P2

Bact.Ind.	TC	FC	FS	FC/FS	CP	SFB
Days	CFU/100mL	CFU/100mL	CFU/100mL		CFU/100mL	CFU/100mL
0	5.3E+03	8.0E+02	1.6E+04	0.05	n.d.	< 1
10	2.5E+03	3.6E+02	1.6E+04	0.02	1.9E+04	< 1
17	1.1E+03	2.2E+02	1.35E+04	0.02	<1	< 1
24	8.2E+02	1.3E+02	1.3E+04	0.01	n.d.	< 1
31	4.2E+02	2.7E+02	1.4E+04	0.02	2.8E+04	< 1
38	4.0E+02	7.0E+01	9.6E+03	0.01	1.2E+04	< 1
45	2.7E+02	< 1	2.5E+03	0.00	2.5E+04	< 1
52	3.2E+02	< 1	1.9E+03	0.00	3.2E+04	< 1
59	3.0E+01	< 1	2.0E+03	0.00	3.8E+04	< 1
66	< 1	< 1	2.1E+03	0.00	1.5E+05	< 1

n.d. = not determined

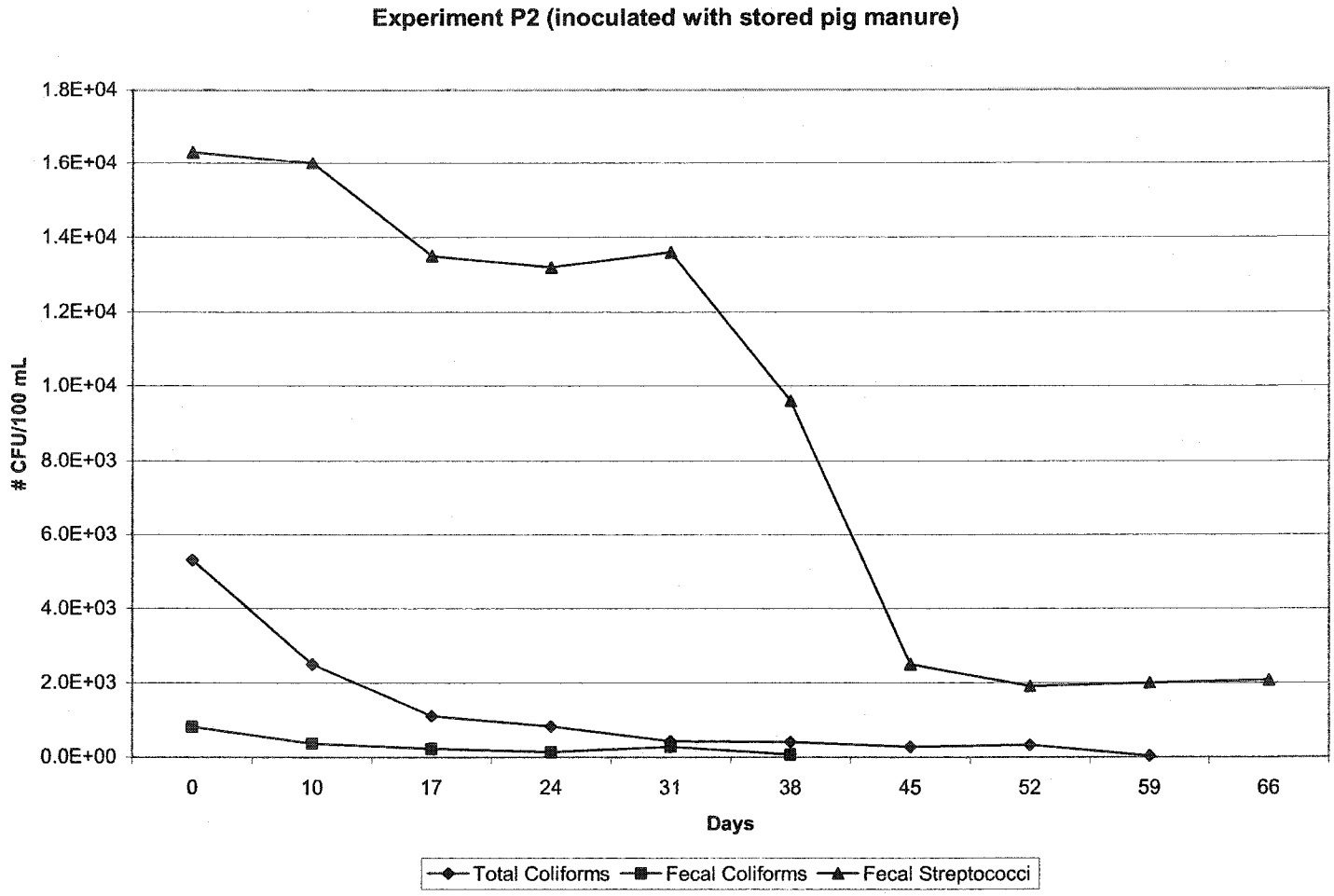


Figure 4.3-a – Decay of BI in Experiment P2 with time.

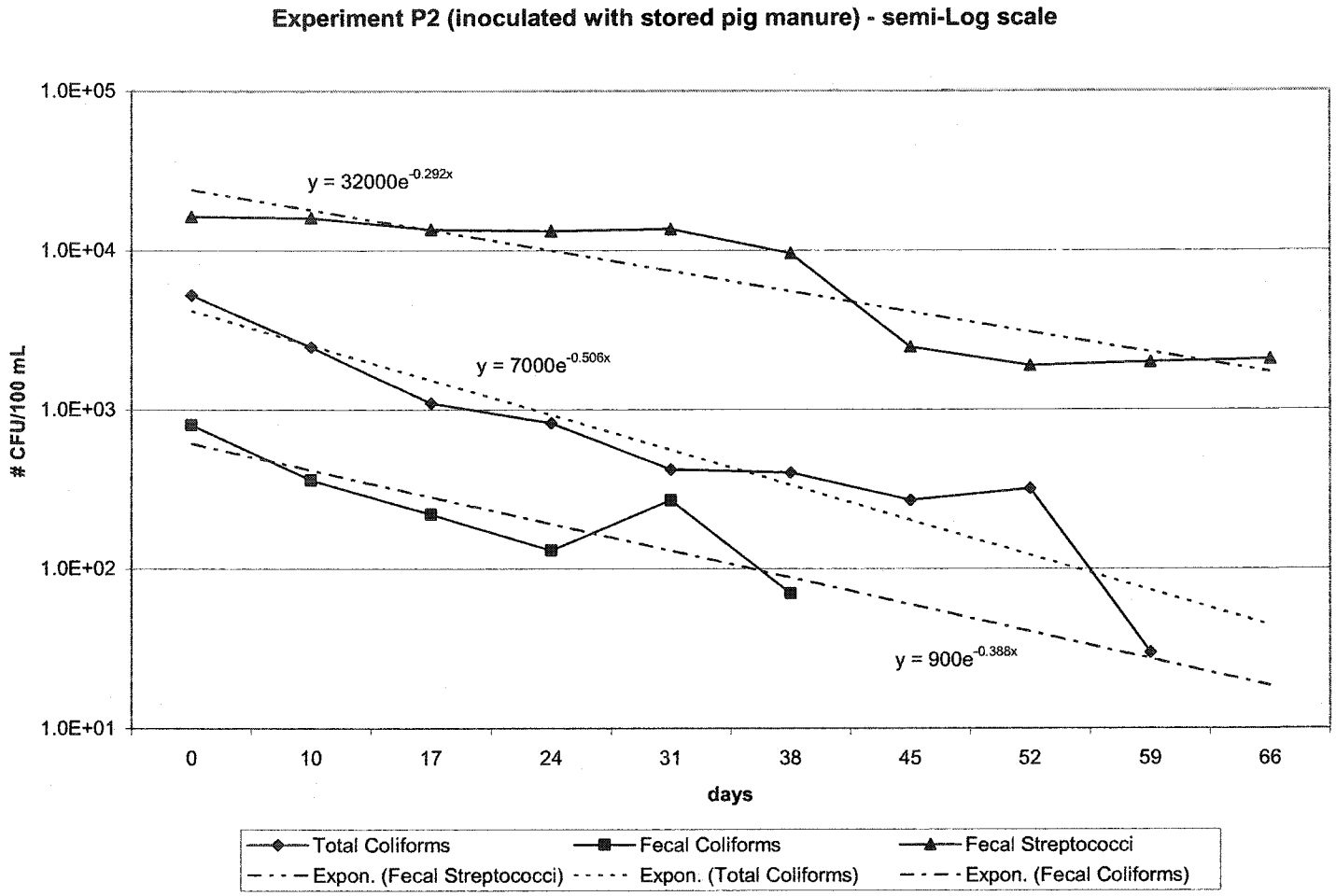


Figure 4.3-b – Decay of BI in Experiment P2 with time and exponential fitting in semi-Log scale.

The results obtained for Experiment S are reported in Table 4.4 and represented in Figure 4.4.

Table 4.4 – Concentrations of BI for microenvironment S

Bact.Ind.	TC	FC	FS	FC/FS	CP	SFB
Days	CFU/100mL	CFU/100mL	CFU/100mL		CFU/100mL	CFU/100mL
0	2.4E+03	6.0E+02	2.0E+04	0.03	8.0E+02	2.8E+03
10	1.6E+03	1.2E+02	1.4E+04	0.01	5.0E+02	9.0E+02
17	2.9E+02	3.8E+01	7.4E+03	0.01	n.d.	< 1
24	1.0E+01	1.0E+00	2.2E+02	0.00	6.5E+02	< 1
31	1.0E+00	< 1	1.1E+02	0.00	3.6E+02	< 1
38	2.0E+00	< 1	2.6E+02	0.00	1.4E+02	< 1
45	2.0E+00	< 1	2.4E+01	0.00	2.8E+02	< 1
52	< 1	< 1	1.0E+01	0.00	4.8E+02	< 1
59	< 1	< 1	1.0E+00	0.00	2.0E+02	< 1
66	< 1	< 1	< 1	0.00	4.6E+02	< 1

n.d. = not determined

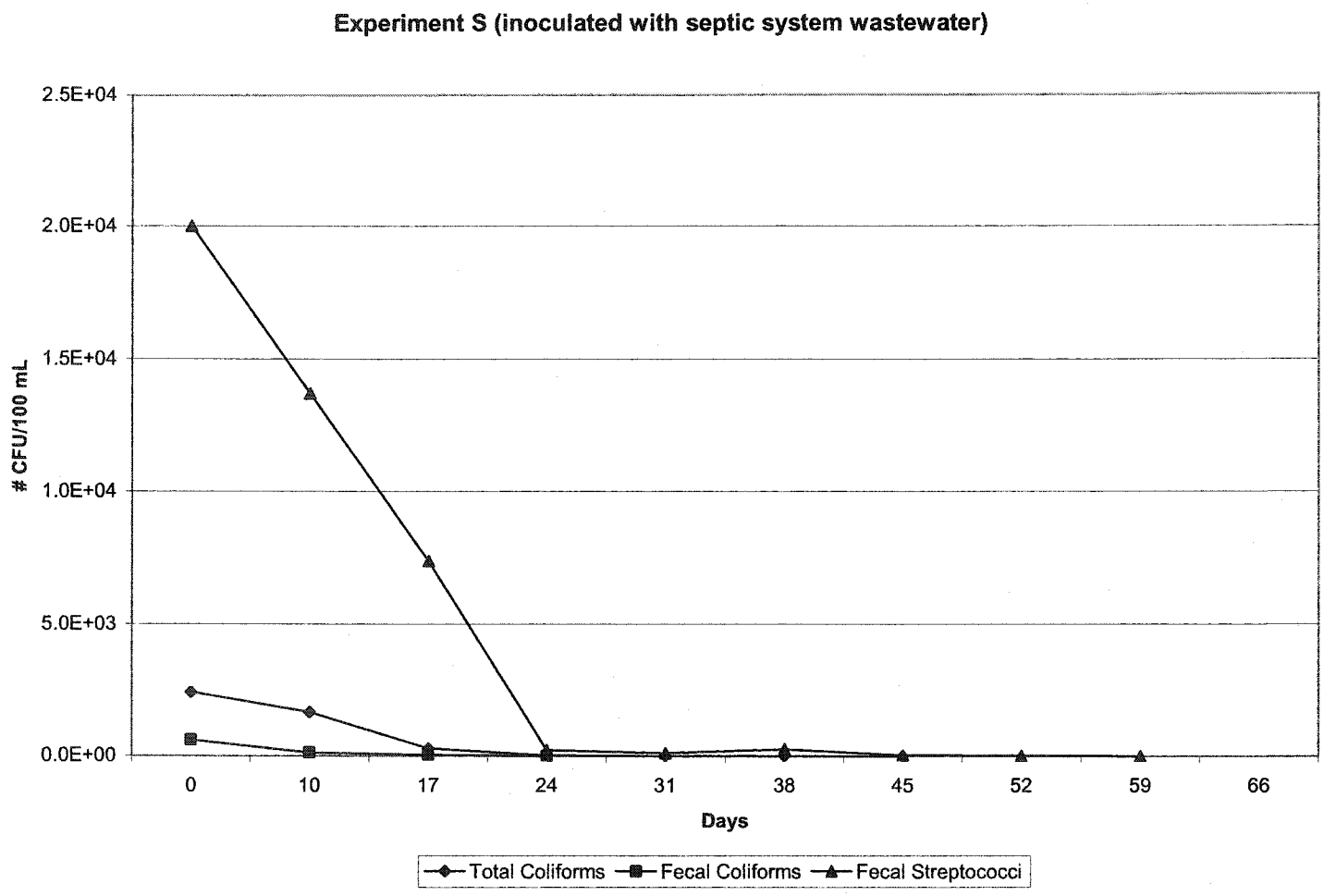


Figure 4.4-a – Decay of BI in Experiment S with time.

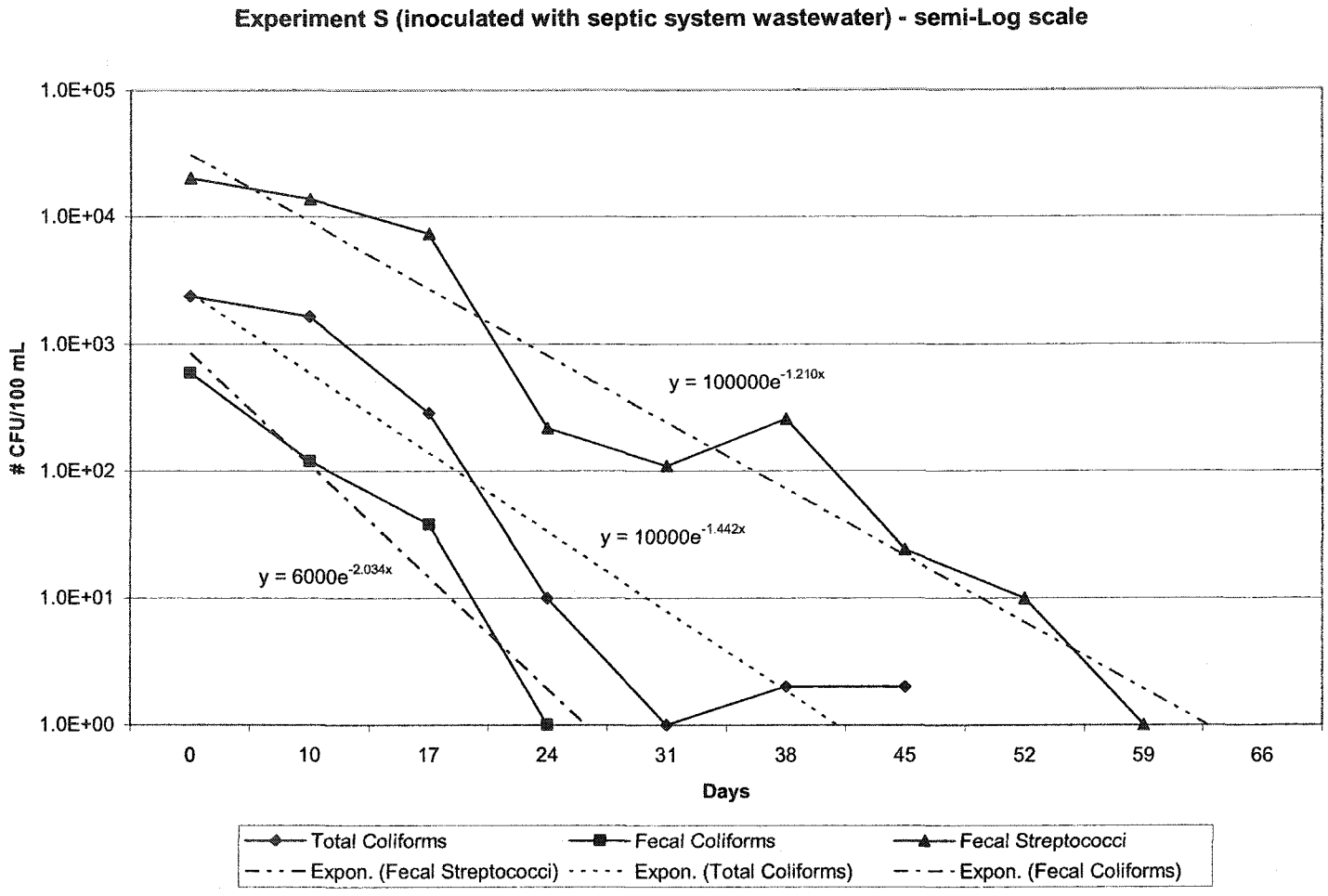


Figure 4.4-b – Decay of BI in Experiment S with time and exponential fitting in semi-Log scale.

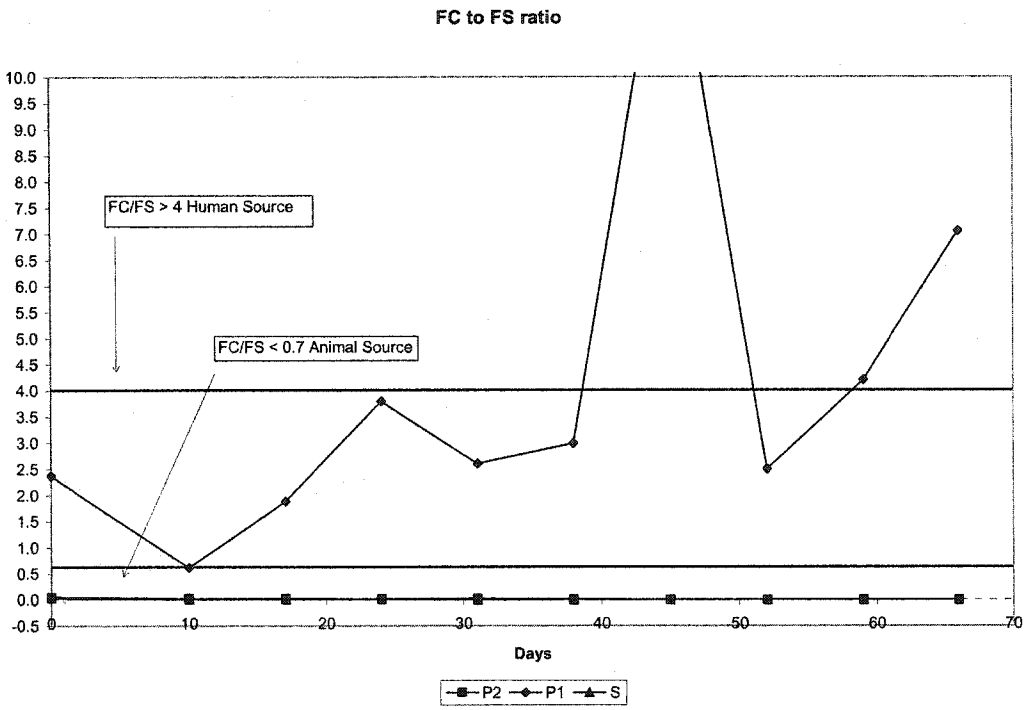


Figure 4.5 – Profile of FC/FS for P1, P2 and S

The behavior of FC/FS versus time is shown in Figure 4.5. The decay of *C. perfringens* with time in Experiments P1, P2 and S are represented in Figure 4.6. Certain values are reported in the tables as "n.d.". In these cases, it was not possible to enumerate the BI because the plates were contaminated by molds. Similarly, the decay of SFB with time in Experiments P1, P2 and S are represented in figure 4.7. In this case the BI were not detected in samples of animal origin (Experiments P1, P2).

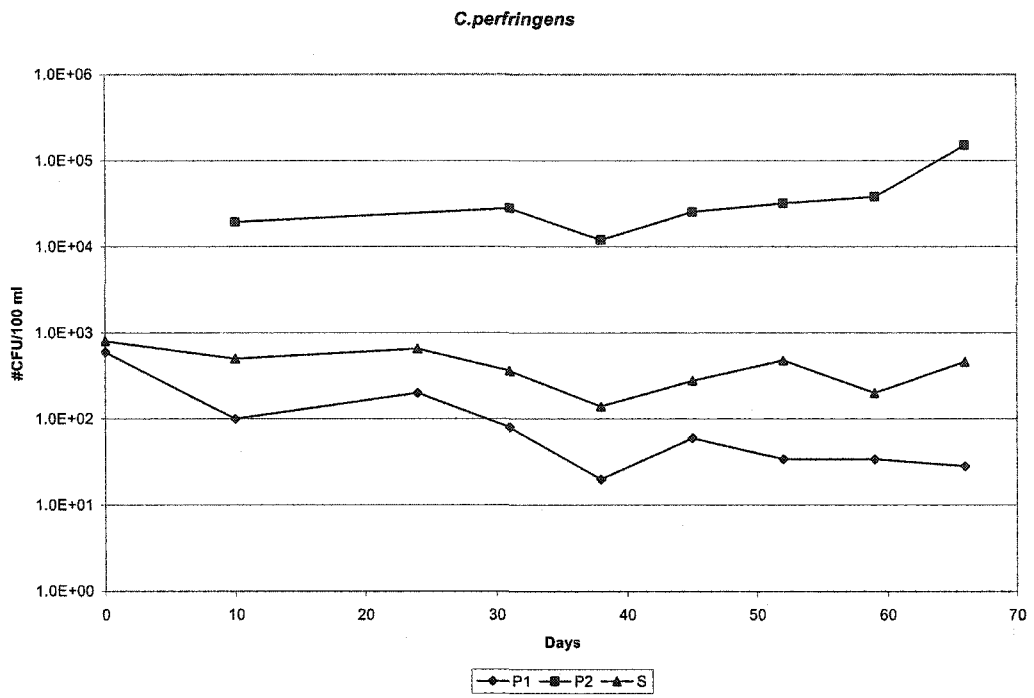


Figure 4.6 – Decay of CP with time in Experiments P1, P2 and S

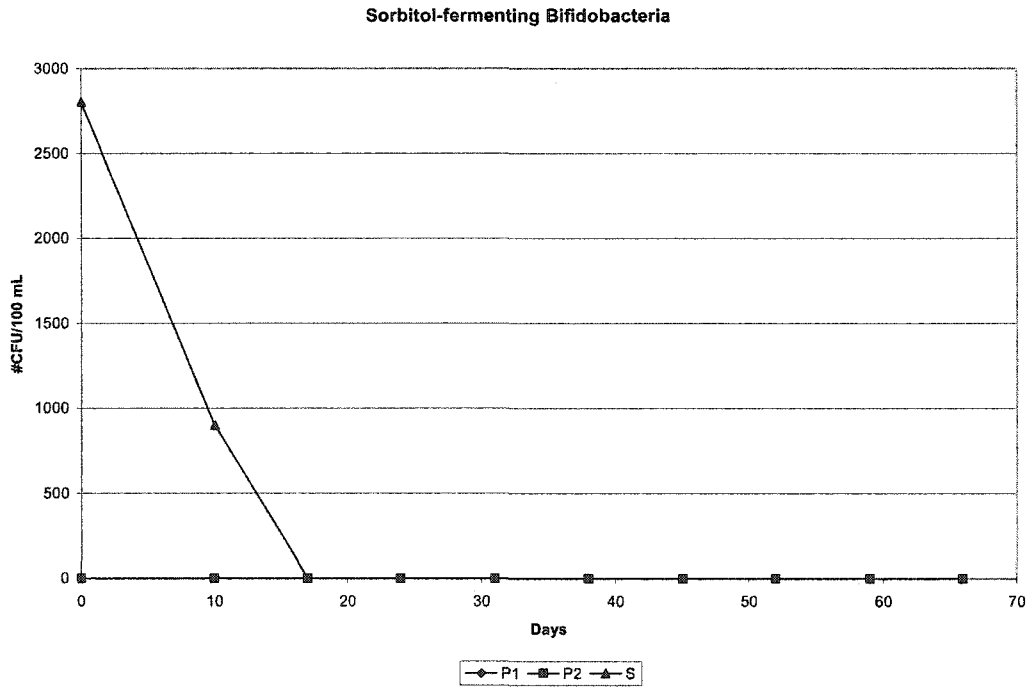


Figure 4.7 – Decay of SFB with time in Experiments P1, P2 and S

In **Experiment P1** (flask inoculated with fresh pig manure), it was observed that the initial concentration of TC was higher than FC, and the concentration of FC was higher than that of FS (TC > FC > FS). A similar trend was observed in Phase I for sample A (dilution of fresh pig manure). In fact, the two samples came from the same place (Mr. Armstrong's pig farm) and were taken at the same time. According to Gerba (Chap. 20 in Maier, 2000), the usual average concentration of FS in pig feces should be higher than TC and FC. There is no plausible explanation for this discrepancy. Probably, the microflora in the intestine of the animals from which the samples were taken was different from those analyzed by Gerba, because of differences in diet, or there may generally be a high variability in the microflora of pig feces.

The die-off coefficients of FC and FS were estimated by assuming that the disappearance of the viable microbes followed a pseudo first-order decay model: $Y=Y_0\exp(-k\cdot t)$, where Y is the concentration of bacteria [# CFU/L] at the time t, Y_0 is the initial concentration, k is the die-off coefficient [d^{-1}], and t is the time [d]. Table 4.5 shows the die-off coefficients values which are quite similar to those reported in literature (see Table 2.1).

Table 4.5 – Estimated Die-off coefficients for the BI

Sample	TC		FC		FS	
	k_{TC}	R^2	k_{FC}	R^2	k_{FS}	R^2
P1	0.267	0.93	0.029	0.04	0.203	0.54
P2	0.506	0.88	0.388	0.75	0.292	0.83
S	1.442	0.86	2.043	0.86	1.207	0.94

The average values found in literature for k_{FC} and k_{FS} are respectively: 0.139 – 0.069; 0.099 – 0.013 (see Table 2.1; values multiplied by 0.434).

The FC to FS ratio was in the range 0.7 - 4 (the average value was 4.2) for most of the samples, except for those taken towards the end of the experiment, when the values were higher than 4. Therefore FC/FS wouldn't have been a reliable method to distinguish the source of fecal pollution in this case (FC/FS < 0.7 for animal sources). This reason of this discrepancy may be due to the anomalous ratios of TC, FC and FS found in these specific samples.

CP was found in all the samples of Experiment P1, and its average concentration was 100 CFU/100 mL. The plate corresponding to the sample taken on the 17th day was discarded because of mold contamination.

SFB were not detected in this experiment, but other white-grey furry unidentified colonies grew on the HBSA plates (see Figure 4.8 - a).

In **Experiment P2** (flask inoculated with manure from a cesspit), it was found that the initial ratios of BI were consistent with those indicated in the literature. FS were almost 2 orders of magnitude greater than FC, and 1 order of magnitude greater than TC (FS ~ 10 TC ~ 100 FC), indicating an "aged" manure.

The die-off coefficients estimated both for FC and FS are higher than those reported in the literature (see Table 4.5).

In this experiment the FC to FS ratio was less than 0.7 for all the samples. Therefore, the application of the FC/FS would have given the right indication of the source of contamination. However, the ratio had decreased with time, and it behaved opposite to what was assumed by Geldreich (see FC/FS shift in Sect. 2.5 – I.a).

In this experiment, CP was always found, and its average concentration was $4 \cdot 10^{+4}$ CFU/100mL. The plates corresponding to the sample taken on the 1st and 24th day were discarded because of mold contamination. SFB were never detected in this experiment, but other blue and brown unidentified colonies grew on the HBSA plates (see Figure 4.8 - b).

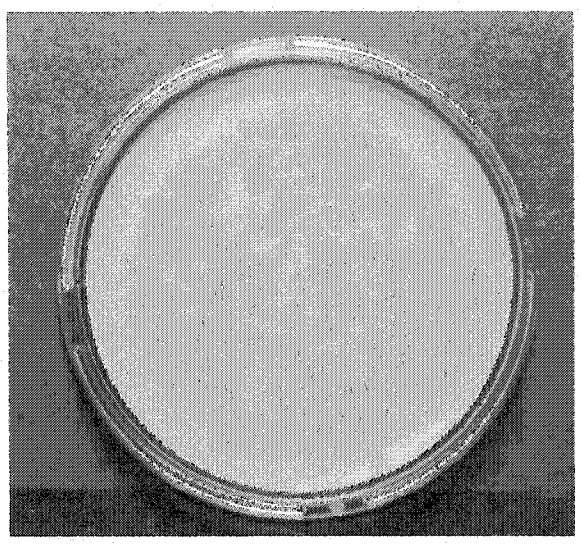
In **Experiment S** (flask inoculated with wastewater from septic system) a trend similar to that observed in Experiment P2 was found for the initial ratio of BI. The concentration of FS was higher than that of TC and FC, (FS ~ 0.1 TC ~ 0.05 FC), again indicating "aged" bacteria in the wastewater.

In this experiment, the die-off coefficients estimated for all BI were much higher than those reported in the literature (see Table 4.5). There are not enough data to explain this abnormal behavior. Probably, the presence of inhibiting substances, e.g. detergents, drugs or other personal care products, was the reason for their fast disappearance. The FC to FS ratio in this case is not a reliable method to identify the source of fecal contamination. In fact, the ratio was never higher than 0.1, indicating an animal rather than a human source of microbial pollution.

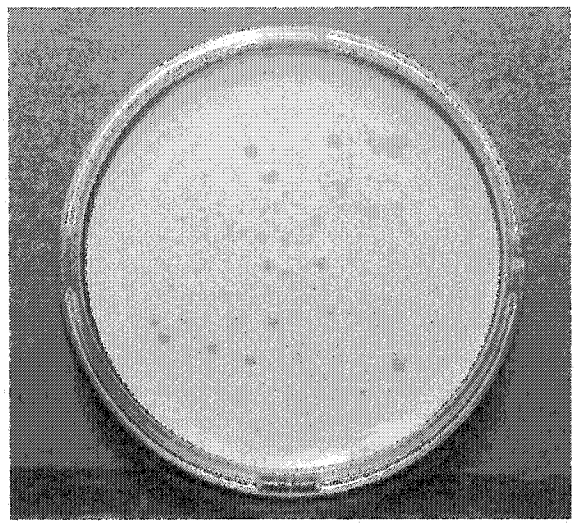
CP was detected in all the samples taken over the period of sampling. The average concentration was $4 \cdot 10^{+2}$ CFU/100mL. The plate corresponding to the sample taken on the 17^{th} day was discarded because of mold contamination.

SFB were detected only in this experiment. Their initial concentration was $2.8 \cdot 10^{+3}$ CFU/100 mL; however, they disappeared after a couple of weeks. Other interfering colonies were observed on the HBSA plates after the disappearance of SFB colonies.

According to the results obtained, the use of SFB as a MST method to distinguish human from animal sources of fecal contamination appears to be the most reliable method of the three tested. Nevertheless, to completely assess the method, the persistence of this BI in a typical groundwater environment should be evaluated.



(a)



(b)

Figure 4.8 – Bacterial species interfering with SFB in Experiments P1 (a) and P2 (b).

4.3 – Phase III

In this phase, three experiments were performed in duplicate (six flasks representing each a GSME experiment). Each flask was inoculated with one of the following pure bacterial cultures:

- *Clostridium perfringens* – pure species (labeled **Cp** α and **Cp** β)
- *Bifidobacterium adolescentis* – pure species (labeled **Ba** γ and **Ba** δ)
- *Sorbitol-fermenting Bifidobacteria* – isolated from sample C in Phase I (labeled **SFB** α and **SFB** β)

Samples of water from each flask were collected in triplicate once every week over a period of no more than 50 days. In Baγ and Baδ the sampling frequency was every 2-3 days. The concentrations of the pure species were determined and the results obtained are presented in the Tables 4.6 to 4.11 and Figures 4.9 to 4.14.

Tables 4.6 and 4.7 and Figures 4.9 and 4.10 show the results for the *C. perfringens* experiments; Tables 4.8 and 4.9 and Figures 4.11 and 4.12 show the results for the *B. adolescentis* experiments; Tables n. 4.10 and 4.11 and Figures 4.13 and 4.14 show the results for the Sorbitol-fermenting Bifidobacteria isolate experiment. In experiments SFB α and SFB β (Sorbitol-fermenting Bifidobacteria) there were problems in the enumeration of the colonies growing in the HBSA plates. These anomalies are discussed in the following paragraphs.

Table 4.6 – Concentrations of *C. perfringens* in "Cp α"

	Sample I	Sample II	Sample II	Average
Days	CFU/100mL	CFU/100mL	CFU/100mL	CFU/100mL
0	1.1E+05	9.5E+04	8.30E+04	9.6E+04
7	1.1E+05	8.1E+04	8.0E+04	9.0E+04
12	1.3E+05	9.0E+04	8.0E+04	1.0E+05
14	8.0E+04	8.0E+04	1.10E+05	9.0E+04
21	8.5E+04	5.5E+04	7.5E+04	7.2E+04
28	9.5E+04	6.0E+04	5.5E+04	7.0E+04
35	7.0E+04	9.0E+04	8.0E+04	8.0E+04
42	8.2E+04	6.7E+04	5.0E+04	8.2E+04
49	8.0E+04	6.7E+04	7.2E+04	8.0E+04

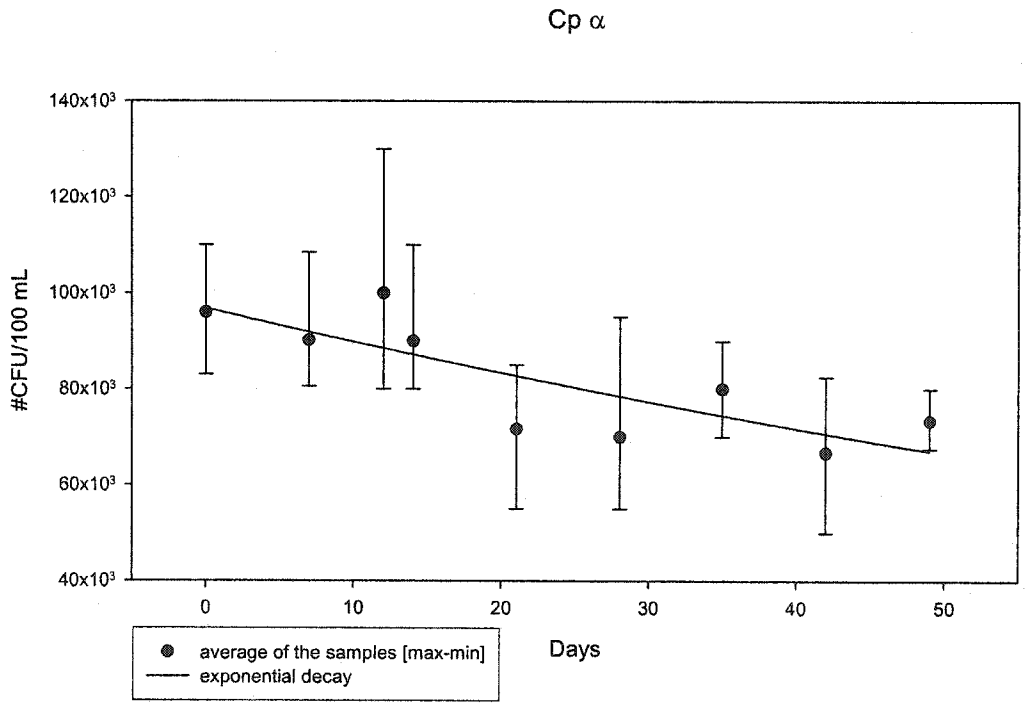


Figure 4.9 – Concentration of C. perfringens with time in Cp α

Table 4.7 – Concentrations of *C. perfringens* in "Cp β"

	Sample I	Sample II	Sample II	Average
Days	CFU/100mL	CFU/100mL	CFU/100mL	CFU/100mL
0	2.8E+05	2.4E+05	2.4E+05	2.5E+05
7	2.2E+05	2.2E+05	2.5E+05	2.3E+05
12	1.9E+05	2.8E+05	3.0E+05	2.6E+05
14	2.8E+05	1.8E+05	2.5E+05	2.4E+05
21	1.8E+05	3.3E+05	2.0E+05	2.4E+05
28	2.0E+05	1.4E+05	2.0E+05	1.8E+05
35	1.7E+05	1.7E+05	1.0E+05	1.5E+05
42	1.8E+05	1.8E+05	1.7E+05	1.8E+05
49	1.2E+05	1.7E+05	1.6E+05	1.5E+05

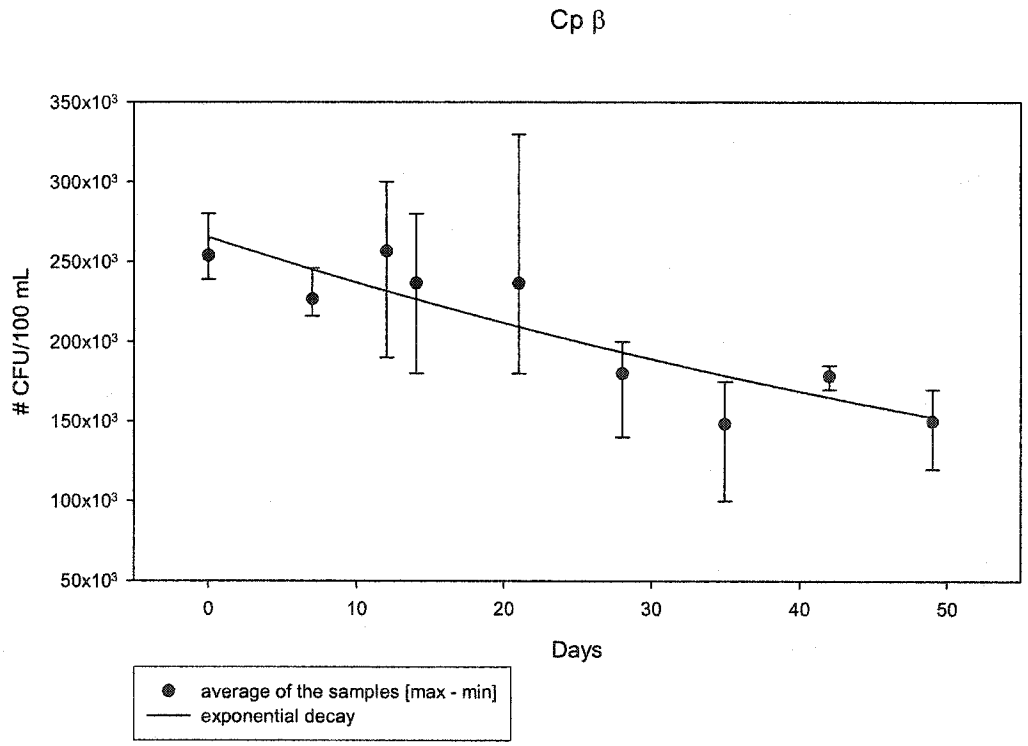


Figure 4.10 – Concentration of C. perfringens with time in Cp β

Table 4.8 – Concentrations of *B. adolescentis* in "Ba γ"

	Sample I	Sample II	Sample II	Average
Days	CFU/100mL	CFU/100mL	CFU/100mL	CFU/100mL
0	1.0E+08	n.d.	n.d.	1.0E+08
2	9.0E+07	9.1E+07	9.3E+07	9.1E+07
4	8.6E+07	8.1E+07	9.3E+07	8. 7E+07
6	8.5E+07	8.5E+07	8.8E+07	8.6E+07
9	8.4E+07	8.9E+07	7.9E+07	8.4E+07
11	8.2E+07	7.8E+07	8.9E+07	8.3E+07
16	7.1E+07	7.7E+07	8.4E+07	7.7E+07
18	6.2E+07	7.7E+07	7.9E+07	7.3E+07
22	6.6E+07	7.5E+07	6.1E+07	6.7E+07
26	6.9E+07	5.5E+07	5.2E+07	5.9E+07
30	5.6E+07	5.2E+07	5.6E+07	5.5E+07

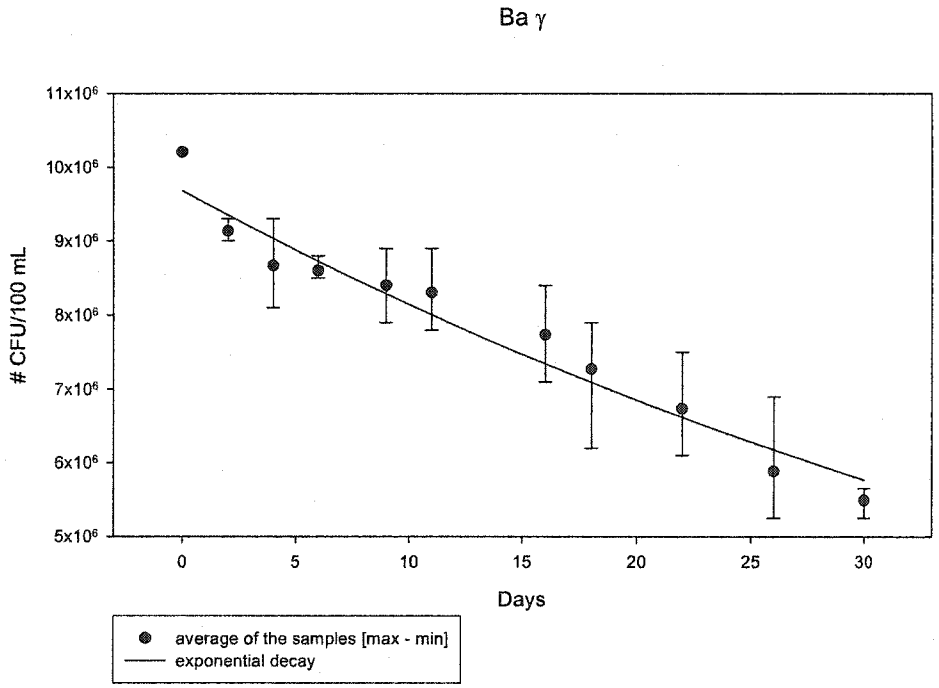


Figure 4.11 – Concentration of B. adolescentis with time in Ba γ

Table 4.9 – Concentrations of *B. adolescentis* in "Ba δ"

	Sample I	Sample II	Sample II	Average
Days	CFU/100mL	CFU/100mL	CFU/100mL	CFU/100mL
0	9.8E+07	n.d.	n.d.	9.8E+07
2	9.0E+07	8.5E+07	9.4E+07	8.9E+07
4	7.7E+07	8.1E+07	7.5E+07	7.8E+07
6	7.3E+07	7.3E+07	8.6E+07	7.7E+07
9	7.9E+07	7.7E+07	8.0E+07	7.9E+07
11	6.2E+07	6.7E+07	5.9E+07	6.3E+07
16	6.2E+07	6.2E+07	7.0E+07	6.5E+07
18	6.7E+07	7.0E+07	6.5E+07	6.7E+07
22	5.6E+07	6.3E+07	5.7E+07	5.9E+07
26	4.8E+07	4.7E+07	3.2E+07	4.2E+07
30	3.1E+07	3.6E+07	3.7E+07	3.5E+07

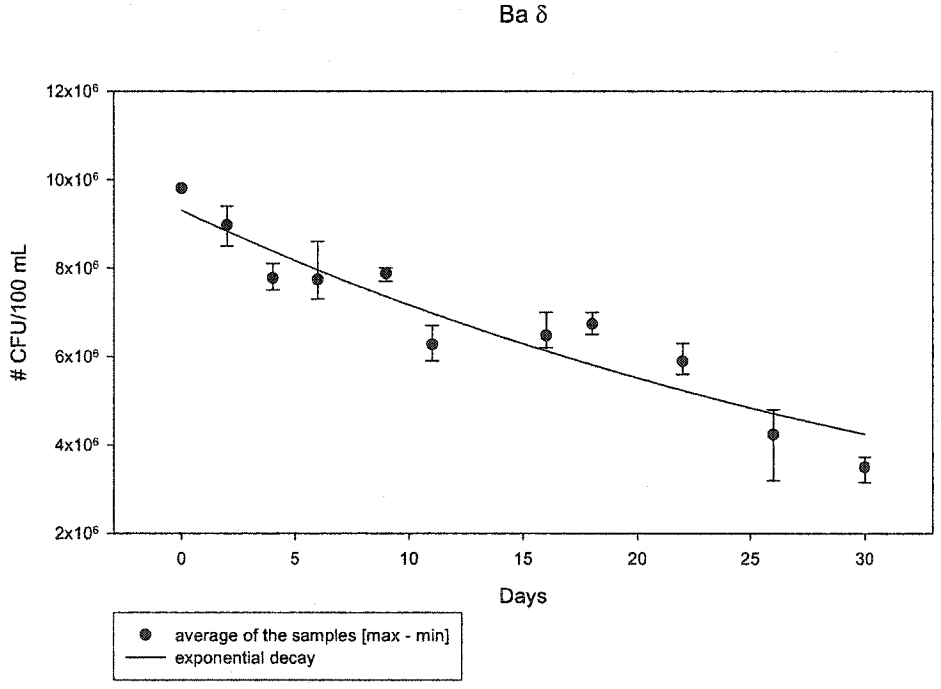


Figure 4.12 – Concentration of B. adolescentis with time in Ba δ

Table 4.10 – Concentrations of SFB in "SFB α"

	Sample I	Sample II	Sample II	Average
Days	CFU/100mL	CFU/100mL	CFU/100mL	CFU/100mL
0	8.3E+06	6.7E+06	6.7E+06	7.2E+06
5	6.3E+06	5.6E+06	6.0E+06	5.9E+06
9	3.8E+06	4.5E+06	4.5E+06	4.3E+06
14	4.4E+06	5.0E+06	5.1E+06	4.8E+06
21	2.0E+06	2.0E+06	1.8E+06	1.9E+06
28	< 1	< 1	< 1	< 1
35	< 1	< 1	< 1	< 1
42	< 1	< 1	< 1	< 1

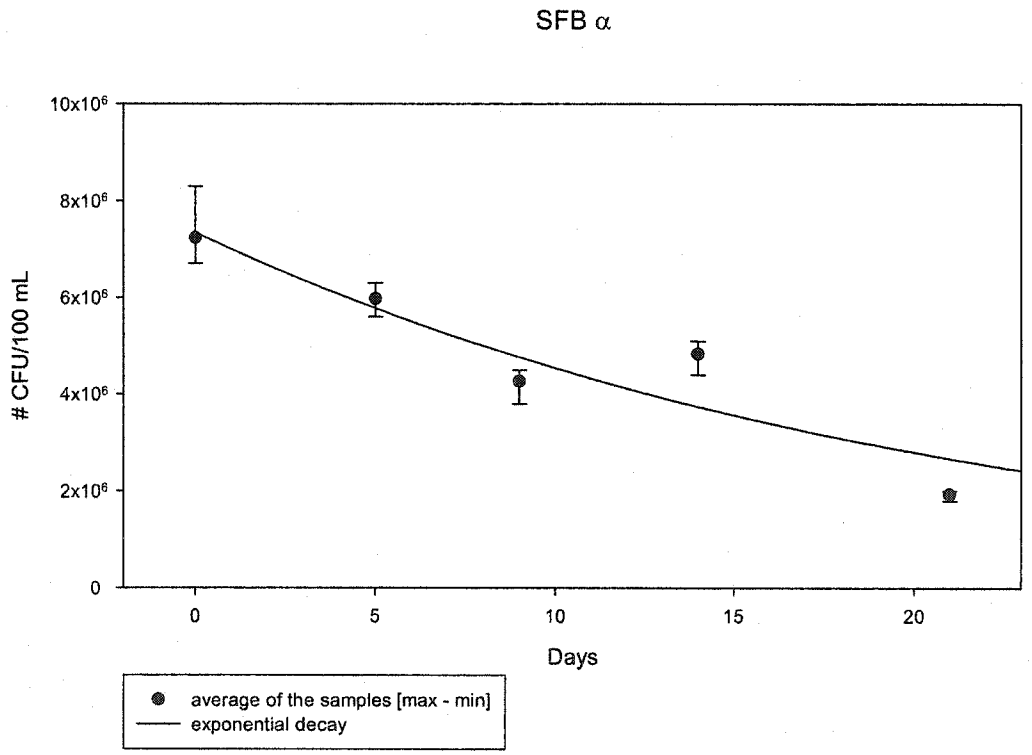


Figure 4.13 – Concentration of SFB with time in SFB α

Table 4.11 – Concentrations of SFB in "SFB β"

	Sample I	Sample II	Sample II	Average
Days	CFU/100mL	CFU/100mL	CFU/100mL	CFU/100mL
0	3.3E+06	4.8E+06	4.4E+06	4.2E+06
5	4.6E+06	4.6E+06	3.0E+06	4.1E+06
9	5.3E+06	3.6E+06	4.7E+06	4.5E+06
14	3.1E+06	3.5E+06	4.0E+06	3.5E+06
21	1.9E+06	1.2E+06	1.6E+06	1.6E+06
28	< 1	< 1	< 1	< 1
35	< 1	< 1	< 1	< 1
42	< 1	< 1	< 1	< 1

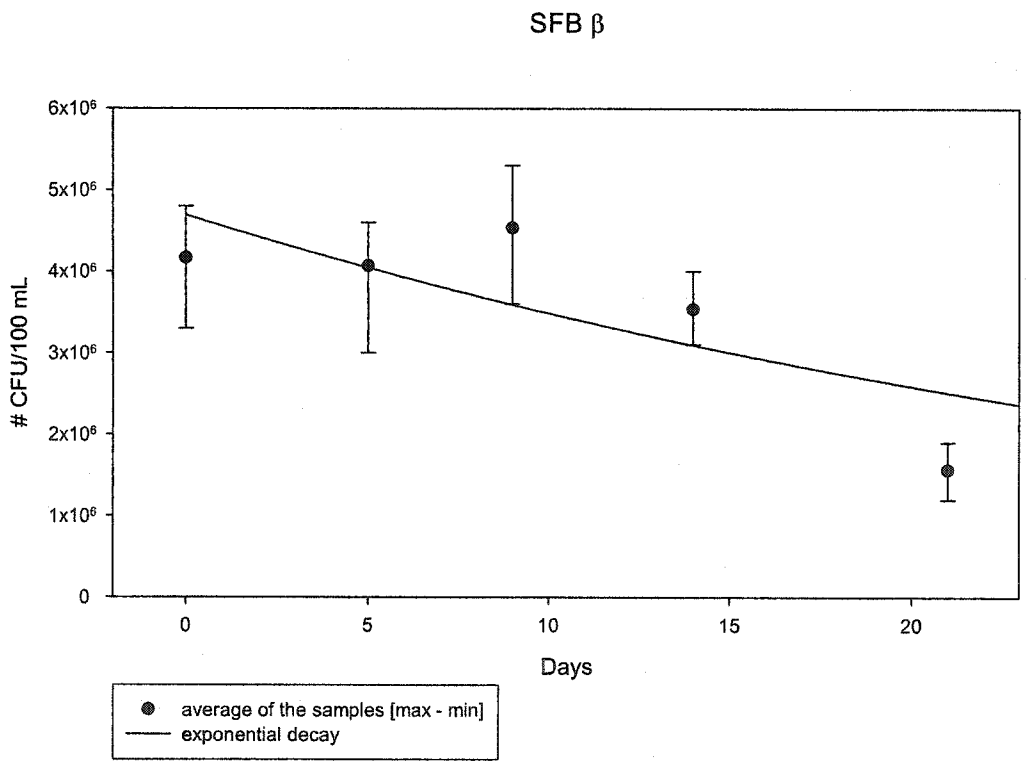


Figure 4.14 – Concentration of SFB with time in SFB β

It was observed that ***C. perfringens*** had an average die-off coefficient of 0.094 d^{-1}, which is very close to that reported in the literature for river waters (0.085; Madema, 1997). It appears that the different environmental conditions in this study did not influence the survival of this species, which is a spore-forming and therefore very resistant to different environmental conditions. The values of the die-off coefficients and the respective R^2 are reported in Table 4.12.

It has been observed that ***B. adolescentis*** can survive quite long in water under the GSME conditions adopted. Because there are not analogous studies available in literature, the die-off coefficient can only be compared to the typical values observed for other bacteria (see Table 2.2). In this case *B. adolescentis* had a die-off coefficient greater than that of *Fecal Streptococci* (0.013). The values of the die-off coefficients and the respective R^2 are reported in Table n. 4.12.

In case of **Sorbitol-fermenting Bifidobacteria,** there were problems in the enumeration, which were related to a contamination of the inoculum. In this case, a regular decay of SFB was observed for the first 15 days. During this period only yellow colonies (SFB) were detected on the plates. However, on the 21st day, other blue colonies were observed together with SFB (see Figure 4.15), and after that time no more SFB colonies were observed. The identification of the blue colonies was unsuccessfully attempted using the BBL Crystal Anaerobe ID Kit. The only information obtained on this species is that they were facultative anaerobic bacteria. The

die-off coefficient was calculated considering only the first 21 days of experiment. The values obtained are slightly lower than those obtained for *B. adolescentis* pure species, and are reported in Table 4.12.

As stated earlier, it was decided to measure the die-off coefficient for SFB also because the strain of *B. adolescentis* supplied by ATCC was susceptible to one of the antibiotics contained in the HBSA medium. In this case, it was assumed that SFB would have been more representative of the real fecal pollution. The presence of another species in the SFB experiments was definitely caused by a contamination occurred during the phases of culturing of the inoculum.

Although the goal of measuring the die-off coefficient failed, still this experiment provided some interesting information regarding the recovery of SFB on the HBSA medium in presence of other competing bacteria. The short persistence of SFB is apparent, and the difficulty in detecting them was probably caused by the presence of other competing species that displace SFB in HBSA agar. The selectivity of HBSA deserves further investigation.

Table 4.12 – Estimated Die-off coefficients for Phase III

BI	Experiment	k [d^{-1}]	R^2
Cp	Ref.	0.008[vii]	-
	α	0.007	0.27
	β	0.011	0.45
Ba	Ref.	n.a.	-
	γ	0.017	0.82
	δ	0.026	0.83
SFB	Ref.	n.a.	-
	α	0.048	0.83
	β	0.030	0.45

n.a. = not available.

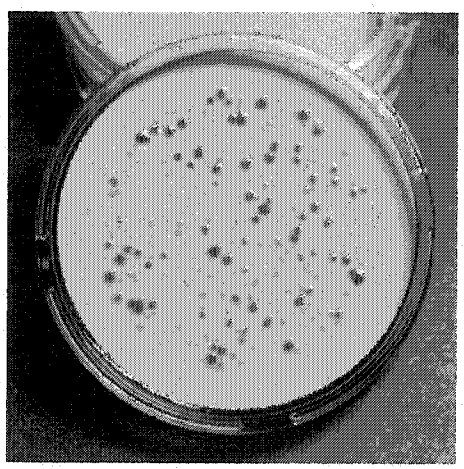


Figure 4.15 – Unknown bacterial colonies competing with SFB in HSBA

[vii] Average value of 0.012 (@ 5°C) and 0.027 (@ 15 °C) (Medema, 1997); values multiplied by 0.434.

4.4 – Phase IV

In this phase, the selected three MST methods were tested on samples of groundwater. The samples were taken from the two wells located in rural areas (Dr J. Laird's well, labeled L; Mr. E. Manzocco's well, labeled M), at two different times (August '03, labeled 1; November '03, labeled 2).

Total Coliforms (TC) and certain chemical parameters were also measured together with the already mentioned bacterial indicators. The results are presented in the Table 4.13. The chemical characteristics of the water samples together with other information concerning the wells are reported in Appendix A7.

Table 4.13 – Results of Phase IV

Bact.Ind.	**TC**	**FC**	**FS**	**FC/FS**	**CP**	**SFB**
Sample	CFU/100mL	CFU/100mL	CFU/100mL		CFU/100mL	CFU/100mL
L1	< 1	< 1	< 1	n.a.	< 1	< 1
L2	21	< 1	5	0.0	< 1	< 1
M2	< 1	< 1	< 1	n.a.	< 1	< 1

n.a. = not applicable

The only one that showed contamination was L2 (taken for Dr. J. Laird's well in November 2003). In L2, only TC and FS were positive, and CP was not detected, even though it is considered to be the most persistent of

the BI measured. Likewise, TC was detected but not FC, even though they should have a similar persistence in groundwater.

In this case, only FC to FS ratio can be applied and it suggests that the source of fecal contamination was animal. This conclusion is supported also by the high density of FS in pig feces compared to human feces (average density of FS was reported as $84 \cdot 10^{+6}$ per gram in pigs versus $3 \cdot 10^{+6}$ per gram in humans; Gerba in Maier, 2000). This should explain the absence of FC. Nevertheless, the reason for the presence of TC is not clear.

Considering the poor performance obtained for FC/FS method in the previous phases of this study, it is recommended that further investigation for this well should be performed in order to determine with certainty the source of contamination.

Chapter 5

Conclusions and Recommendations

In this study, three different Microbial Source Tracking methods (MST), applicable to distinguish human from animal sources of fecal contamination in groundwater, were evaluated in laboratory experiments. The experiments were conducted in four phases, and the following conclusions are drawn:

5.1 – The objective of the first phase was to identify the most reliable MST method among the three investigated. The results have shown that:

- the most reliable method is the detection of Human Bifidobacteria (SFB);
- the FC to FS ratio method is not always accurate;
- *C. perfringens* cannot be considered a source specific indicator;
- there was a lack of selectivity of the growth medium used for SFB.

5.2 – The goal of the second phase was the observation of the changes of performance with time for the selected MST methods. During this phase, it was observed that:

- the FC/FS method was accurate only in one experiment out of three;
- the Human Bifidobacteria were detected only for a short period (2 weeks) after the inoculation. This fact represents a significant

limitation to this method;

- *C. perfringens* was detected during the entire period of four weeks, testifying its potential reliability as indicator of the timing of contamination, rather than as a source specific indicator.

5.3 – The objective of the third phase was to determine the die-off coefficients for *C. perfringens* and *B. adolescentis* (the main component of the group SFB) in simulated groundwater. It was observed that:

- the values of the die-off coefficient obtained for *C. perfringens* were in accordance with those available in the literature for surface waters;
- *B. adolescentis* was sensitive to one of the antimicrobials present in the recovery medium. Therefore, the medium was modified in order to measure the survival of this species. The die-off coefficient measured with this method resulted smaller than expected;
- the experiment attempted with SFB, accidentally contaminated by another species, demonstrated that the short persistence observed for SFB in the previous experiments was probably only apparent;
- from the results obtained in Phases I, II and III for SFB it can be inferred that HBSA is probably not enough specific for Bifidobacteria to guarantee always reliable results in tracking sources of fecal contamination in groundwater.

5.4 – In Phase four, the use of the MST methods was attempted in the field. Although a certain degree of contamination was observed in one of the

samples, only the FC/FS method, which is not always reliable, was applicable.

5.5 – In summary, the use of Sorbitol-fermenting Bifidobacteria demonstrated to be a reliable indicator of human sources of fecal contamination in all the experiments performed; but, this method can be applied only in case of recent contamination events. The problems encountered in this study suggest that HSBA (the growth medium used for the detection of SFB) is not enough selective and specific of Bifidobacteria.

5.6 – Recommendations: further investigations are recommended for this BI, by trying other growth media or by modifying the one used in this study. For example, it is suggested to isolate Bifidobacteria from water samples using membrane filtration with the new BFM agar defined by Nebra (1999), and then to identify the group of Sorbitol-fermenting colonies among the Bifidobacteria growing in the BFM-plate by using a presence/absence test with HBSA as growth broth.

Considering the results obtained in Phase IV, it is recommended, to use one of the phenotypic or chemical MST methods described in the literature review in order to identify unequivocally an animal source of fecal contamination in groundwater. For example, in the specific case of pig manure contamination, it is suggested the use of Antibiotic Resistance Analysis (ARA) or the use of the antibiotics adopted as growth promoters as chemical tracers.

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Appendices

A1 – Media Preparation

A1.1 – m-Endo Total Coliforms broth

The medium was supplied by Millipore Corporation under the catalogue number MB000000E. For the preparation of the medium, the producers indicated the following procedure:

- Prepare a sterile solution of deionized water containing 2% ethanol (CH_3CH_2OH 95%).
- Add 4.8 g of dehydrated product in 100 mL of solution.
- Mix carefully and heat to boiling point.
- Immediately remove when boils.

During this study, the medium produced some insoluble black suspension. Therefore, the prepared medium was let cool and the solids precipitate; after that, the clarified medium was transferred in a new sterile bottle and stored in a refrigerator @ 4 °C for no longer than two weeks.

The Petri dishes were prepared by adding about 2 mL of m-Endo over an adsorbent pad about 1 hour before the use.

A1.2 – m-FC Fecal Coliforms broth

The medium was supplied by Millipore Corporation under the catalogue number MB000000F. For the preparation of the medium, the producers indicated the following procedure:

- Suspend 3.7 g of dehydrated product in 100 mL of deionized water.

- Add 1 mL of Rosolic Acid, solution 1% in 0.2 N NaOH.
- If necessary, adjust pH to 7.4 with HCl 1N.
- Heat to boiling point.

The prepared medium was cooled and stored in a refrigerator @ 4 °C for no longer than two weeks.

The Petri dishes were prepared by adding about 2 mL of m-Endo over an adsorbent pad about 1 hour before the use.

The solution of rosolic acid was prepared by adding 1 g of powder (Millipore Corporation, catalogue number MB000000F) in 100 mL of a sterile solution of 0.2 N of NaOH.

A1.3 – KF Streptococcus Agar

The medium was supplied by Millipore Corporation under the following catalogue number MB000000S. For the preparation of the medium the producers indicated the following procedure:

- Suspend 7.64 g of dehydrated product in 100 mL of deionized water.
- Boil to dissolve completely.
- Heat for 5 minutes at boiling point and then remove and let cool.
- Add 1 mL of Triphenyltetrazolium Chloride solution 1 % at a temperature of 50-60 °C.
- Mix well and pour into plates immediately.

The prepared plates were preserved in plastic bags and stored in a

refrigerator @ 4 °C for no longer than four weeks.

The solution of Triphenyltetrazolium Chloride (TTC) was prepared adding 1 g of product (Millipore Corporation, catalogue number MB0000TTC) in 100 mL of deionized water.

A1.4 – SFP Agar + Egg Yolk enrich. + Polymyxin B Sulfate + Kanamyxin Sulfate

The Chemicals necessary to prepare the medium were supplied by Difco (Becton Dickinson) under the catalogue numbers: SFP Agar Base 500 g, 0811-17; Antimicrobic Vial K 6 x 10 mL, 3339-60; Antimicrobic Vial P 6 x 10 mL, 3268-60; Egg Yolk Enrichment 50% 12 x 10 mL, 3347-61. For the preparation of the medium, the producers indicated the following procedure:

- Suspend 4.7 g of dehydrated product (base) in 90 mL of deionized water.
- Mix thoroughly.
- Heath with agitation until boiling point.
- Boil for 1 minute.
- Autoclave at 121 °C for 15-20 minutes.
- Cool at 50 – 60 °C.
- Add 1/10 [1 mL] of rehydrated vial antimicrobial P (= 3000 IU of Polymyxin B Sulfate).
- Add 0.48 mL of rehydrated vial antimicrobial K (= 1.2 mg of Kanamyxin Sulfate).
- Add 10 mL of egg yolk enrichment 50 %.
- Mix well and pour into plates immediately.

The prepared plates were preserved in plastic bags and stored in a refrigerator @ 4 °C for no longer than four weeks.

It should be noted that the addition of 10 mL of egg yolk enrichment was done after adding the two antimicrobials in order to avoid excessive cooling, thereby creating difficulty in pouring to the Petri plates.

A1.5 – mCP Agar modified

Because this medium was not available in the market, it was prepared by following the guidelines give in the literature (Armon, 1988).

The composition of the base was the following:

- Polypeptone BBL 30.0 g
- Yeast Extract 20.0 g
- Sucrose 5.0 g
- L-cysteine Hydrochloride 1.0 g
- $MgSO_4$ $7H_20$ 0.1 g
- Bromcresol Purple 0.04 g
- Agar 15.0 g

The preparation was done in the following steps:

- Add the above indicated ingredients to 900 mL deionized water in a 2 L Erlenmeyer flask.
- Stir and heat to dissolve in a boiling water bath.
- Bring the pH to 7.6 with 1 N NaOH.

This base was stored aseptically in a refrigerator @ 4 °C. Each time it was needed, 90 mL of this base was measured and autoclaved for 15 minutes at 121 °C, and allowed to cool at 50-60 °C. The medium was completed adding aseptically and mixing well the following components:

- D-cycloserine 40 mg
- Polymixin B sulfate 2.5 mg
- 4.5 % $FeCl_3 \cdot 6H_2O$ solution 200 µL
- 0.5 % Phenolphthalein diphosphate solution 2.0 mL
- 0.075 % Indoxyl-β-D-glucoside solution 8.0 mL

After that, the medium was mixed well and poured immediately into plates. The prepared plates were preserved in plastic bags and stored in a refrigerator @ 4 °C for no longer than four weeks.

A1.6 – Human Bifido Sorbitol fermenting Agar (HBSA)

Because this medium was not available in the marked, it was prepared by following the guidelines give in the literature (Mara, 1983).

The composition of the base was:

- Sorbitol 10.0 g
- Polypeptone 10.0 g
- Yeast Extract 20.0 g
- Casamino Acids 8.0 g
- Sodium Chloride 3.2 g
- Bromcresol Purple 0.1 g

The preparation was done in the following steps:

- Add deionized water up to 1000 mL to the above indicated ingredients in a volumetric flask.
- Stir and heat to dissolve at boiling temperature for 5-10 minutes.
- Cool.

Then, the following ingredients were added:

- Cysteine Hydrochloride 0.4 g
- Nalidixic Acid 30 mg
- Agar 15 g

The pH was adjusted to 6.9 ± 0.1 by using solution 0.1 N HCl. This base was stored aseptically in a refrigerator @ 4 °C. Each time it was needed, 94 mL of this base was measured and autoclaved for 15 minutes at 121 °C, and allowed to cool at 50-60 °C. Te medium was completed by adding aseptically and mixing well the following components:

- solution 1000 IU/mL of Polymixin B sulfate 1 mL
- solution 1 mg/L of Kanamyxin sulfate 5.0 mL

(Equivalent to a concentration of 1000 IU/mL Polymyxin B sulfate and of 50 µg/mL kanamyxin sulfate)

After that, the medium was mixed well and poured immediately into plates. The prepared plates was preserved in plastic bags and stored in a

refrigerator @ 4 °C for no longer than four weeks.

A2 – Confirmation Tests

A2.1 – Iron Milk medium preparation

The medium, used for confirmation of C. perfringens, was prepared according to the procedure described in literature (EPA/600/R-95/178, US EPA, 1996).

The composition of 1 L of medium was:

- Fresh pasteurized, homogenized milk (3.5% butterfat) 1.0 L
- $FeSO_4 \cdot 7H_2O$ 1.0 g

For the preparation, the steps were:

- Dissolve ferrous sulfate in 50 mL water.
- Add slowly to 1 L milk and mix with magnetic stirrer.
- Dispense 11 mL of medium into culture tubes.
- Cap and autoclave 12 min at 118 °C.

The prepared medium was stored aseptically in a refrigerator @ 4 °C for no longer than three weeks.

A2.2 – Clostridium perfringens confirmation procedure

U.S.EPA (EPA/600/R-95/178, US EPA, 1996) recommended the following procedure for the confirmation test of *C. perfringens* in water and groundwater samples.

- Pick at least 10 typical isolated *C. perfringens* colonies from the

mCP plate and transfer each into a separate tube containing a non selective clostridial medium. Incubate at 35 C for 24 h. Examine by gram stain and for purity. *C. perfringens* are short gram-positive bacilli. Retain tubes for further testing.

- Inoculate ten tubes of iron milk medium with 1 mL from the ten tubes and incubate in a 44.5 C water-bath for two h. Examine hourly for stormy fermentation with rapid coagulation and fractured rising curd.
- Those colonies which are gram-positive, non-motile, and produce stormy fermentation of milk in these confirmatory tests are considered confirmed *C. perfringens*.

Pink or magenta colonies counted on mCP medium are adjusted to a count/100 mL and reported as presumptive *C. perfringens* colony forming units (CFU)/100 mL. The presumptive count is normally used for routine monitoring. If confirmation tests are performed, original counts on mCP agar are adjusted based on the percent of colonies picked and confirmed (# of positives/# of total colonies). Multiply the previous concentration by this ration and report as confirmed *C. perfringens* CFU/100 mL of water sample.

U.S.EPA suggests the use of Fluid Thioglycollate Medium (Difco, Cat # 0432-02-6) as non selective medium for the growth of the isolates to be confirmed. In this study, Reinforced Clostridial medium (Difco, Cat. # 1808-17) was used as non-selective medium. This medium is recommended as the ideal growth medium for *C. perfringens* by ATCC.

A2.3 – Confirmation of anaerobic isolates using BBL Crystal™ Identification System

A rapid identification kit was used to identify some species interfering in the enumeration of C. perfringens and Sorbitol fermenting Bifidobacteria, and also as a confirmation test,. The kit, know as *BBL Crystal™ Identification System – Anaerobe ID Kit*, is a miniaturized identification method employing modified conventional, fluorogenic and chromogenic substances, intended for the identification of frequently isolate anaerobic bacteria. It is used mainly in diagnostic medicine, but it can also identify, among all the species, also *C. perfringens* and *B. adolescentis*, *B. breve*, *B. infantis*, which are Sorbitol fermenting Bifidobacteria, the typically human Bifidobacteria species. The BBL Crystal anaerobe ID System also requires Gram stain, catalase and indole test results in order to give an accurate result.

For the use of this kit, the following procedure was adopted:

- Identification of a colony of interest in a selective medium (SFP, mCP or HSBA).
- Expansion of the colony in a tube containing Reinforced Clostridial medium (the colonies were picked up by using a sterilized cotton swab, successively immersed in the tube).
- Incubation of the tube for 1 day at 37 °C.
- The isolate was successively spread on a Blood-agar plate, and incubated at anaerobic conditions for 2 days at 37 °C.
- Colonies from the Blood-agar plate were picked up and used for the identification according to the specific procedures given by

the producers of the kit.

The passage of the colonies through the Blood-agar was necessary because the database, given by the producers, refers to that kind of medium.

Gram Staining Test

The Gram stain is a test used to identify bacteria. The Gram stain allows classifying bacteria in Gram-positive or Gram-negative, and is a technique discovered at the end of the 19th century.

The staining technique consists of the following steps:

- Application of primary stain (crystal violet)
- Application of mordant (Gram's iodine) that combines with the crystal violet in the cell forming the crystal violet-iodine
- Application of a decolorizing agent (ethyl alcohol), in this step the primary stain is washed our (decolorized) of some bacteria, while others are unaffected
- Application of the secondary stain or counter-stain (safranin), which stains the decolorized bacteria red.

The key factor in the procedure is that bacteria differ in their rate of decolorization, those that decolorize easily are referred to as gram-negative, whereas those that retain the primary stain are called gram-positive.

The complete procedure is outlined here:

1. Preparation of the smear: make a few circles with a loop

immerged in the sample tube on the clean slide.

2. Preparation of the Gram stain:
   - Cover the smear with crystal violet and leave it for 30 seconds.
   - Wash the slide with distilled water avoiding direct squirting.
   - Cover the smear with Gram's iodine for 10 seconds.
   - Wash of the iodine with water.
   - Decolorize with 95 % ethyl alcohol, washing the smear until no large amounts of purple wash out.
   - Wash gently with water.
   - Add safranin for 30 seconds.
   - Wash the slide with water and blot with a paper towel.

3. Examination of the smear under the microscope.

Indole Test

One of the end products from bacterial oxidation of the amino acid is tryptophan. This amino acid can be oxidized by some bacteria to form three major end products: indole, pyruvic acid, and ammonia. Kovacs Reagent is used to detect the presence of indole and it has the following composition:

- p-dimethylaminobenzaldehyde 50 g
- amyl or butyl alcohol 750 mL
- HCl (conc.) 250 mL

Detection of indole indicates tryptophan degradation and can be accomplished by the addition of certain aldehydes to form colored end

products. The active ingredient in Kovacs Reagent reacts with indole to form a pinkish-red end product that is highly visible. The indole test is performed on cultures grown in broth media containing a suitable amount of tryptophan. When Kovac's reagent is added to the culture tube, it forms a layer above the media. If the layer has no color the reaction is negative. In a positive reaction, the Kovac's reagent turns red.

To perform the indole test add 4 to 6 drops of Kovacs reagent in a culture tube. mix gently and observe for the development of color.

Catalase test

Some bacteria and macrophages can reduce diatomic oxygen to hydrogen peroxide or superoxide. Both of these molecules are toxic to bacteria. However, some bacteria possess a defense mechanism which can minimize the harm done by these two compounds. These resistant bacteria use two enzymes to catalyze the conversion of hydrogen peroxide and superoxide back into diatomic oxygen and water. One of these enzymes is catalase and its presence can be detected by a simple test. The catalase test involves adding hydrogen peroxide to a culture sample or agar slant. If the bacteria in question produce catalase, they will convert the hydrogen peroxide and oxygen gas will be evolved. The evolution of gas causes bubbles to form and is indicative of a positive test.

The procedure for this simple test is the following:

- Pick the inoculum from a culture and place it on a clean and sterilized slide.

- Add one or two drops of H_2O_2 (3% hydrogen peroxide solution).
- Observe for the development of bubbles.

Often lots of bubbles develop rapidly on the slide; even slight bubbles indicate positive reaction.

A3 – Susceptibility Test

The Susceptibility test is done in order to determine whether a microorganism is susceptible to a specific antibiotic, which means that the growth of the organisms is affected negatively by the presence of the antibiotic. There are a number of laboratory (in vitro) tests for the determination of the susceptibility, among which are Kirby-Bauer method, Schlichter test, E test, and the traditional agar and broth dilution methods (Christofilogiannis, 2001; Rosenblatt, 1989; EUCAST, 1992). The objective of all these tests is the determination of the minimum inhibitory concentration (MIC). The MIC is the minimum concentration of the antibacterial agent in a given culture medium below which bacterial growth is not inhibited.

In this study, the Broth Dilution method was used for MIC determination. In this procedure, dilutions of antibiotic are incorporated in the broth. Each dilution tube is inoculated with a standard inoculum of the test organism. After appropriate incubation, the first tube showing macroscopic inhibition of growth is considered the MIC. Tubes showing no visible growth are sub-cultured to agar medium to determine whether or not the organism was inhibited or killed. The minimum bactericidal concentration (MBC) is that concentration in which subculture has no colonies growing.

This method consists of three main phases: preparation of dilutions, inoculation,; determination of MIC.

- Preparation of dilutions: From the antibiotic stock solution, a series

of dilutions are prepared in broth to yield concentrations twice the desired final concentrations. In a macrobroth dilution test, the final volume in each test tube is usually 1 mL (0.5 mL of antibiotic dilution in broth and 0.5 mL of bacterial inoculum in broth).

- Preparation of inoculum: Suspend a loopful of bacteria (from 5-10 colonies) in 1 mL of PBS or saline, yielding a suspension corresponding to the turbidity of McFarland 0.5. Take 50 µL to 5 mL broth (approx. 106 CFU/mL). Use this suspension, within 30 min to inoculate tubes (0.5 mL/tube) already containing the antibiotic dilutions in broth, resulting in a 1/2 dilution of each concentration and of the bacterial inoculum. Incubate the inoculated tubes or trays at 35-37 °C for 16-20 h (or longer if necessary) in the recommended atmosphere for the bacterial species to be tested. As control, always include a reference strain with known MIC in parallel with the test strain.
- Reading MIC: Shake the tubes gently before reading. It is recommended to judge the turbidity in the tubes against a dark background. The MIC is read as the lowest concentration yielding no visible growth.

The tubes not presenting visible growth can be checked to determine the MBC.

McFarland turbidity Standards

McFarland turbidity standards are used as a reference standard to approximate the number of bacteria in a liquid suspension. Reproducible and meaningful results can be obtained by challenging identification and/or susceptibility testing procedures with a standardized inoculum,. Failure to use a standardized amount of inoculum in some test situations, including antimicrobial susceptibility testing procedures and some identification kits, will lead to erroneous results.

Each standard can be prepared by mixing the appropriate volume of a 1.175 % Barium Chloride with a 1% solution of sulfuric acid, in the proportions indicated in the following Table A3.1.

Table A3.1 – Preparation of McFarland Standards

McFarland Standard #	Vol. (mL)		Number of bacteria/mL ($x10^8$)
	(1.175%) $BaCl_2 \cdot 2H_2O$	(1%) H_2SO_4	
0.5	0.5	99.5	1.5
1	1.0	99.0	3
2	2.0	98.0	6
3	3.0	97.0	9
4	4.0	96.0	12
5	5.0	95.0	15
6	6.0	94.0	18
7	7.0	93.0	21
8	8.0	92.0	24
9	9.0	91.0	27
10	10.0	90.0	30

The procedure for the preparation of the inoculum using McFarland standard 0.5 (or 4 for the BBL Crystal kit) is the following:

- Mix McFarland Reference Standard well inverting the tube a number of times.
- With adequate lighting, visually compare turbidity of McFarland Standard to that of the test suspension; To assist in viewing suspensions, hold both tubes in front of a Wickerham card or newspaper print.
- Add isolated colonies to uninoculated broth or saline test tube; Mix well and compare to McFarland Standard.
- If it is easier to view lines on Wickerham card (or text on newsprint) through the test suspension than through the McFarland standard, then the suspension is too light. Continue to add one colony at a time until suspension is equivalent to reference standard.
- If it is harder to view lines on Wickerham card (or text on newsprint) through the test suspension than through the McFarland standard, then the suspension is too heavy; Dilute the test suspension with sterile buffer solution until the test suspension is equivalent to the reference standard.

A4 – Bacterial culturing and isolation procedures

The two species used as indicator, plus other isolates obtained from the selective plates, were cultivated using Reinforced Clostridial medium in sterilized borosilicate tubes. The preparation of the culture tubes was done with the usual aseptic techniques. The inoculation of the tubes was done using the following methods:

- When the inoculum was liquid broth, the inoculum bacteria were transferred to the new tube by micro-pipetting 0.5 – 1 mL of medium in the new tube (using sterilized tips).
- When the sample was a colony isolated in a plate containing a selective medium, e.g. HBSA or SFP agar plates, the inoculum colony was transferred by using a cotton-swab previously sterilized of a metal loop sterilized by flame.

The typical selectivity of the specific media utilized was used for the isolation of the colonies. Then the colonies were identified by color, general morphology and consistency of the colonies. In dealing with anaerobic species, the guidelines given by Levett (1991) and Board (1992) were considered.

A5 – Sterilization Techniques

Of the many the typical methods of sterilization available (see Chapter 7 in Tortora, 2004), the following procedures were used in this study:

- Autoclaved at 121 °C and 103.4 kPa for 20 minutes, for media, general glassware (flasks, bakers etc...), pipettes, filters and also for bio-hazardous wastes
- Exposed to non-ionizing radiation (UV light at 250 nm), particularly for the plastic funnel tops of the filtration units. The exposure time was usually 5-10 minutes
- Used 95 % Ethanol solution, particularly for the non autoclavable parts of the micropipettes, for the lab table and for the dilution flasks' stoppers
- Exposed to flame, particularly for the forceps and glass sticks.

All these operations were performed using aseptic criteria.

A6 – Procedure for dilution of samples

For the membrane filtration technique, the procedure described in "Experiment 10" by Gerba (in Pepper, 1995) was adopted. Whenever the number of bacteria was higher than ~ 6E+3 # CFU/100 mL, the water sample was diluted using Phosphate Buffered Dilution Water (PBDW). The PBDW was prepared by adding 1.25 mL of stock phosphate buffer solution and 5 mL of magnesium chloride solution to sterilized water in volumetric flasks of 1000 mL. The stock phosphate buffer solution was prepared by dissolving 34.0 g of potassium dihydrogen phosphate (KH_2PO_4) in 500 mL of deionized water. After adjusting the pH to 7.2 with a solution 1 N of NaOH, the volume was brought to 1 L, and the solution was autoclaved at 121 °C for 20 minutes. The magnesium chloride solution was prepared by dissolving 81.4 g of hexahydrate magnesium chloride ($MgCl_2 \cdot 6H_2O$) in 1 L of deionized water. The solution prepared was then autoclaved.

The dilutions were obtained according to the Table A6.1.

Table A6.1 – Dilutions for high concentrations of CFU

Sample (S)	Dilution (D)	Volume of D filtered
0.1 mL	1 mL of S + 999 mL PBDW (D1)	100 mL of D1
0.01 mL	1 mL of S + 999 mL PBDW (D1)	10 mL of D1
1 µL	1 mL of S + 999 mL PBDW (D1)	1 mL of D1
0.1 µL	10 mL of D1 + 990 mL PBDW (D2)	10 mL of D2
0.01 µL	10 mL of D1 + 990 mL PBDW (D2)	1 mL of D2

A7 –Characteristics of the wells and of the ground water samples

A7.1 – Dr Laird's well

Location and surrounding areas: the well is located in Blenheim, in the municipality of Chatham-Kent, about 2 km north of the Rondeau Bay and the shore of Lake Erie. The only buildings close to the well are the house and the farm of Dr. Laird, which is occupied only during the weekends. About 800 m north there are few other houses, about 600 m east there is a pig farm, and in the other directions there are vineyards and fields.

Characteristics of the soils in the area: it was not possible to retrieve detailed information about the soil stratification, but, according to the owner the area around this farm there has a first layer of gravel about 10 m deep, after which there is a consistent layer of clay. The well was dug in an aquifer which can be supplied directly by surface run-off (no upper impermeable soil layers). Therefore, it appears to be very vulnerable to surface contamination.

Well characteristics: 7.5 - 10 m of depth; no information about its construction; probable age more than 50 years.

Chemical characteristics of groundwater: only certain parameters were determined and the values are reported in Table A7.1

A7.2 – Mr. Manzocco's well

Location and surrounding areas: the well is located in Essex, in the countryside, about 2 km south-west of the North Ridge. There are no

buildings in the surrounding area. The closest construction is a farm located about 600 m north-east from the well. In the other directions, there are only fields and woods.

Characteristics of the soils in the area: according to the well contractor, the area around the well has a first layer of clay about 15 m deep that isolates the aquifer from the surface. After that, there are alternating layers of gravel, sand and clay. The well receiving water from a confined aquifer, and therefore should not be vulnerable to run-off contamination.

Well characteristics: 45 m of depth; drilled in 2003; maximum flow-rate 25 L/min.

Chemical characteristics of groundwater: only certain parameters were determined and the values are reported in Table A7.1

Table A7.1 – Groundwater qualities

Sample	L1	L2	M2	Method	Natural GW (Ellis, 2000)
pH	7.0	7.0	6.8	Inst.	6.9 - 7.2
Hardness [mg $CaCO_3$/L]	530.5.	n.d.	n.d.	2340 B	190 - 230
Temperature [° C]	13.5	10.2	10.8	Inst.	7.0 - 7.2
Turbidity [NTU]	< 0.2	12	< 0.2	2130 B	< 0.2
Suspended solids [mg/L]	< 0.1	14.5	< 0.1	2540 B	n.a.
Dissolved O_2 [mg/L]	< 0.8	< 0.8	< 0.4	Inst.	< 0.2
Conductivity [µS]	369.0	385.5	313.5	Inst.	n.a.
Total Carbon [mg/L]	62.18	67.15	40.25	Inst.	n.a.
Inorganic Carbon [mg/L]	57.75	62.17	35.67	Inst.	n.a.
TOC [mg/L]	4.43	4.98	4.58	Calc.	< 2
Mn [mg/L]	0.38	n.d.	n.d.	3500 – Mn B	1.8 – 2.0
Ca [mg/L]	164.9.	n.d.	n.d.	3500 – Ca B	69 - 75
Mg [mg/L]	28.8	n.d.	n.d.	3500 – Mg B	n.a

Instr. = Direct instrumental measurement

Calc. = Calculated

Vita Auctoris

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